REMARKS

Reconsideration of the above-identified application in view of the amendments above and the remarks following is respectfully requested.

Claims 1-50 are in this case. Claims 1-36, 40-42, 44-47 and 50 were withdrawn under a restriction requirement as drawn to a non-elected invention. Claims 1-36 and 40-50 have now been canceled. Claims 37-39 have now been amended.

35 U.S.C. § 112, Second Paragraph, Rejections

The Examiner has rejected claims 39 and 49 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. The Examiner's rejections are respectfully traversed. Claim 39 has now been amended. Claim 49 has now been canceled, rendering moot the Examiner's rejection of this claim.

With respect to claim 39 the Examiner states that the phrase "in context of a ... cell" in the claims is not clear. Claim 39 has now been amended to recite the limitation of the pharmaceutical composition further comprising, as an active ingredient, an antigen presenting cell, said antigen-presenting cell presenting the peptide of claim 37, to thereby overcome the Examiner's rejection.

35 U.S.C. § 101/112-1, Rejections

The Examiner has rejected claims 37-39, 43, 48 and 49 under 35 U.S.C. § 101 as claiming an invention not supported by a substantial and specific utility, or by a well-established utility, and has as a consequence further rejected these claims under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The Examiner's rejections are respectfully traversed. Claims 43, 48 and 49 have now been cancelled, thereby rendering moot the Examiner's rejection of these claims. Claims 37-39 have now been amended.

In particular, the Examiner states that the SEQ ID NO: 13 peptide (hereinafter the claimed peptide) is shown in Table 9 of the specification not to be specific to any

particular cell type, being present in all types of cells in the Table. The Examiner further states that while the specification indeed asserts that DNA methyl transferase (MTDM), the protein from which the peptide is derived, is expressed in different cancer cells, that there is no showing that this enzyme is specific to cancer cells. The Examiner additionally states that Niederreither *et al.* teach that this enzyme is present in various tissues. Moreover, the Examiner asserts that the specification teaches that only a few out of thousands of MHC-binding peptides may eventually become candidates for development of anti-cancer vaccines and that no particular information about beneficial features of the claimed peptide is present in the specification.

Applicant wishes to respectfully point out that all of the cell types in which the claimed peptide is shown to be present in Table 9 are cancer cells and that the specification indeed clearly states that this enzyme is specifically overexpressed in cancer cells relative to non-cancer cells (specification, page 98, sentence starting on line 7 to end of paragraph; "... *DNA methyl transferase is ... highly expressed in different cancer cell types... [Li, L.C., et al., 2000 Cancer Res. 60:702-6; Szyf, M., 1998. Cancer. Metastasis Rev. 17:219-31; Pilat, M.J. et al., 1998. Anticancer Res. 18:2575-82]. Increased (DNA methyl transferase) activity is usually associated with tumor progression and is considered to be an important event in cell transformation [Szyf, M., 1998. Cancer Metastasis Rev. 17:219-31; Szyf, M., 1996. Pharmacol Ther. 70:1-37]*). The fact that DNA methyltransferase is indeed specifically overexpressed in cancer cells relative to non-cancer cells is taught by the Li *et al.* reference (refer to enclosed article thereof: Abstract, page 704, column 2, third paragraph; page 705, column 2, second-to-last paragraph), the Szyf, 1996 reference (refer to enclosed abstract thereof), the Szyf, 1998 reference (refer to enclosed abstract thereof), and the Pilat *et al.* reference (refer to enclosed abstract thereof) cited in the specification. In addition, the Li *et al.* reference cited in the specification cites Belinsky *et al.*, 1996 (refer to enclosed Abstract thereof) and El-Deiry *et al.* (refer to enclosed Abstract thereof) which both provide further strong evidence that DNA methyltransferase is indeed specifically overexpressed in cancer cells relative to non-cancer cells. At the time of filing, there was yet additional evidence in the literature of the art that DNA methyl transferase is specifically overexpressed in cancer cells relative to non-cancer cells (refer, for example to enclosed abstract of Belinsky *et al.*).

It is therefore clear that, in sharp contrast to the Examiner's assertion, the specification shows that DNA methyl transferase is indeed specifically overexpressed in cancer cells relative to non-cancer cells.

Applicant wishes to further respectfully point out that Niederreither *et al.* teach that DNA methyl transferase may be expressed in non-cancer cells during mouse gestational stages of development and not after gestation. Since cancer is overwhelmingly and inherently a phenomenon which is of concern post-gestationally, it will be obvious to one of ordinary skill in the art that aspects of the present invention relating to cancer cells inherently relate to cancer in a post-gestational stage organism. Hence, the fact that Niederreither *et al.* report that DNA methyl transferase may be expressed in non-cancer cells during gestational stages of development is not relevant to aspects of the instant invention relating to cancer cells which inherently relate to post-gestational stage organisms. Indeed, numerous genes are known to be normally expressed at significant levels only during gestational developmental stages and to be specifically re-activated in cancer cells (refer, for example, to enclosed abstracts of: Biran *et al.*; Baldwin, RW.; and Coggin JH. Jr.). Additionally, Applicant wishes to respectfully point out that Niederreither *et al.* actually teach that DNA methyl transferase is indeed specifically overexpressed in cancer cells relative to non-cancer cells ("*...we report that tumors ...all show elevated ... (DNA methyl transferase) expression specific to the transformed tissue.*", Abstract, final sentence).

Since, according to the overwhelming art evidence described above, the claimed peptide is indeed clearly derived from a protein specifically expressed in cancer cells, and since the claimed peptide can be used according to the teachings of the instant invention, for example, to generate antigen presenting cells presenting the claimed peptide which are capable of activating T lymphocytes specific for the claimed peptide (specification, page 62, second paragraph; page 70, last paragraph until end thereof on page 71; Figure 6), the claimed peptide indeed clearly has utility, in sharp contrast to the Examiner's assertion that it does not. Applicant is furthermore of the very strong opinion that at the time the invention was made, it would have been well within the purview of one of ordinary skill in the art to utilize the claimed peptide in any one of various ways for treating a disease such as cancer characterized by affected cells presenting the claimed peptide, as amply evidenced by the literature

of the art (refer, for example, to enclosed abstracts of: Knutson KL. and Disis ML.; Wiesmuller KH. *et al.*; Jager D. *et al.*; Eisenbach *et al.*; and Sinkovics and Horvath).

Critically, subsequent to the filing of the invention, Applicant has successfully demonstrated the predicted utility of the claimed peptide for generating antigen presenting cells which present the claimed peptide and are capable of activating/clonally expanding CTLs (refer to enclosed manuscript of Berg et al., page 14, second paragraph) that have the capacity to specifically kill tumor cells displaying the claimed peptide (refer to enclosed manuscript of Berg et al., page 15, second paragraph), and secrete interferon-gamma specifically in response to stimulation with cells presenting the claimed peptide (refer to enclosed manuscript of Berg et al., page 16, second paragraph).

In view of the above arguments, Applicant believes to have overcome the 35 U.S.C. § 101/112-1, rejections.

In view of the above amendments and remarks it is respectfully submitted that claims 37-39 are now in condition for allowance. Prompt notice of allowance is respectfully and earnestly solicited.

Respectfully submitted,

Sol Sheinbein
Registration No. 25,457

Date: August 12, 2003

Encl.:
Article of Li, L.C., et al., 2000 Cancer Res. 60:702-6;
Abstract of Baldwin RW., Ciba Found Symp. 1983;96:230-41;
Abstract of Belinsky, S. A. *et al.*, 1995. Toxicol Lett. 82-83:335-40;
Abstract of Belinsky, S. A. *et al.*, 1996. Proc. Natl. Acad. Sci. USA, 93:4045-4050;
Abstract of Biran *et al.*, Med Hypotheses. 1994 Aug;43(2):119-23;
Abstract of Coggin JH Jr., Ciba Found Symp. 1983;96:28-54;
Abstract of Eisenbach et al., Immunol Lett. 2000 Sep 15;74(1):27-34;
Abstract of el-Deiry, W. S. *et al.*, 1991. Proc. Natl. Acad. Sci. USA, 88:3470-3474;

Abstract of Jager D. et al., Oncology. 2001;60(1):1-7;
Abstract of Knutson KL. and Disis ML., Clin Breast Cancer. 2001 Apr;2(1):73-9;
Abstract of Pilat, M.J. et al., 1998. Anticancer Res. 18:2575-82;
Abstract of Sinkovics and Horvath, Int J Oncol. 2000 Jan;16(1):81-96;
Abstract of Szyf, M., 1996. Pharmacol Ther. 70:1-37;
Abstract of Szyf M., 1998. Cancer. Metastasis Rev. 17:219-31;
Abstract of Wiesmuller KH. et al., Biol Chem. 2001 Apr;382(4):571-9;
A declaration by Dr. Eilon Barnea;
Manuscript of Berg M. *et al.*; and
A two-months extension fee.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

BARNEA ET AL.

Serial No.: 09/865,548

Filed: May 29, 2001

Group Art Unit: 1631

For: METHOD OF IDENTIFYING PEPTIDES CAPABLE OF BINDING TO MHC MOLECULES, PEPTIDES IDENTIFIED THEREBY AND THEIR USES

Attorney Docket: 01/22080

Examiner: Michael L. Borin

Commissioner for Patents
P. O. Box 1450 Alexandria VA 22313

DECLARATION OF ARIE ADMON UNDER 37 CFR 1.132

I am presently employed as researcher at the Technion – Israel Institute of Technology, Department of Biology, where I am Associate Professor. I also serve as the head of the Proteomics Center. I received my Ph.D. degree from The Weizmann Institute of Science in 1984, worked as a post-doctoral fellow in the Chemistry Department at Cornell University and in Cold Spring Harbor Laboratory, and was a senior research associate at the Howard Hughes Medical Institute, Department of Molecular & Cell Biology, U.C. Berkeley, California.

My research focuses on proteomics and tumor immunology. Since the beginning of my career, I have published 54 scientific articles in highly regarded journals and books, and have presented my achievements at numerous international scientific conferences.

I am a member of AAA, ASBMB, ABRF, and ASMS.

Refer to my enclosed curriculum vitae for further details.

I am a co-inventor of the subject matter claimed in the above-referenced U.S. patent application.

I have read the Official action issued with respect to the above-identified

application.

In this Official action, the Examiner has rejected claims 37-39 under 35 U.S.C. § 101 as claiming an invention not supported by a substantial and specific utility, or by a well-established utility, and has consequently rejected these claims under 35 U.S.C. § 112, first paragraph, as lacking guidance enabling one skilled in the art to practice the claimed invention.

The experimental results described in the enclosed Berg *et al.* manuscript conclusively show that a peptide having an amino acid sequence set forth by SEQ ID NO: 13 (hereinafter the claimed peptide) can be used for: (i) generating antigen presenting cells which present the claimed peptide and are capable of activating/clonally expanding CTLs (refer to enclosed manuscript of Berg et al., page 14, second paragraph) that have the capacity to specifically kill tumor cells displaying the claimed peptide (refer to enclosed manuscript of Berg et al., page 15, second paragraph), and secrete interferon-gamma specifically in response to stimulation with cells presenting the claimed peptide (refer to enclosed manuscript of Berg et al., page 16, second paragraph). Such results hereby prove that the rejections of claims 37-39 under 35 U.S.C. § 101/112-1 are unfounded.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United states Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

August 11, 2003

Prof. Arie Admon

Enc.:
Manuscript of Berg *et al.*; and
Curriculum vitae of Arie Admon

[CANCER RESEARCH 60, 702–706, February 1, 2000]

Frequent Methylation of Estrogen Receptor in Prostate Cancer: Correlation with Tumor Progression[1]

Long-Cheng Li, Richard Chui, Koichi Nakajima, Bong Ryoul Oh, Harry C. Au, and Rajvir Dahiya[2]

Department of Urology, Veterans Affairs Medical Center, and University of California–San Francisco, San Francisco, California 94121

ABSTRACT

Prior studies have shown that the estrogen receptor (*ER*) gene is down-regulated in prostate cancer, but the mechanism of its inactivation is not known. We hypothesize that inactivation of the *ER* gene in prostate cancer is through promoter methylation. To test this hypothesis, we investigated the methylation status of the *ER* gene in prostate cancer cell lines, prostate cancer, and benign prostatic hyperplasia (BPH) tissues samples using the bisulfite genomic sequencing method. Our results show that the *ER* gene promoter was methylated in 100% (six of six) of the prostate cancer cell lines tested and all were accompanied by loss of *ER* mRNA expression. Treatment of these cell lines with demethylating agent 5-aza-2′-deoxycytidine restored *ER* mRNA expression in all of the *ER*-negative cell lines. In addition, elevated expression of DNA methyltransferase mRNA was found in all of the prostate cancer cell lines. Of the prostate tissue samples analyzed, 60% (6 of 10) in the BPH samples, 80% (8 of 10) in the low-grade cancer samples (grades I and II), and 95% (20 of 21) in the high-grade cancer samples (grades III-V) exhibited promoter methylation of the *ER* gene. The overall methylation levels in the cancer samples were higher than that in the BPH samples. The differences between the high-grade cancer samples and BPH samples were significant at all CpG sites. Only at three CpG sites were the differences significant between the low-grade cancer samples and BPH samples. This study presents the first evidence that *ER* gene is transcriptionally inactivated by DNA methylation in prostate cancer. Our data suggest that *ER* may be involved in the pathogenesis of prostate cancer, as well as BPH.

INTRODUCTION

During the past 50 years, estrogen has been used for the treatment of prostate cancer. Palliative, although not curative, effects have been widely acknowledged (1). It is generally believed that the favorable response to estrogen therapy in hormone-sensitive prostate cancer is mediated primarily via suppression of the hypothalmo-hypophyseal axis, thus reducing the circulating androgens. Mangan *et al.* (2) have proposed a direct action of estrogen on prostate, presumably via the ER.[3] This hypothesis is also supported by several other investigators (3, 4); however, the functional role of estrogenic steroids and *ER* in the prostate is not clear. In recent years, with the demonstration of *ER* in normal, hyperplastic, and cancerous prostate tissue (5, 6) and of stimulating or inhibitory effects of estrogen on *in vitro* growth of prostate cancer cells (7, 8), evidence began to accumulate that estrogen may exert direct effects on prostate via its own receptor.

The *ER* gene is located on chromosome 6q25.1 (9) and belongs to a superfamily of transcription activators (10). Its protein product is a transcription factor that regulates the expression of estrogen-responsive genes by binding to a specific DNA sequence found in their regulatory regions. As a mediator of estrogen hormone action, the *ER* is involved in many important physiological processes. Loss or down-regulation of *ER* expression in prostate cancer has been frequently documented (5, 11, 12). In addition, an inverse correlation was found between *ER* expression and histological grade or pathological stage by Nativ *et al.* (13) and others (14, 15). Low *ER* expression was also associated with poor prognosis for effective endocrine therapy (14). However, the precise role of ER in neoplasmic transformation of prostate has not been established.

No mutation or other gross structural alterations of the *ER* gene in prostate cancer has been reported thus far to be responsible for *ER* down-regulation. One mechanism that could block transcription of the *ER* gene in *ER*-negative prostate cancer, without structural alteration in the gene, is the *de novo* methylation of cytosine-rich areas, termed "CpG islands," in the 5′ regulatory region of the gene (16). To date, the DNA Mtase encoded by *Dnmt 1* is the only enzyme that has been shown to cause increased CpG dinucleotide methylation (17) and to trigger transformation (18). *ER* gene methylation has been observed in several human cancers, such as breast cancer (19), lung cancer, colorectal cancer, (20) and hematopoietic neoplasms (21), and has been related to inactivation of *ER* expression. The purpose of this investigation was to determine whether *ER* methylation is involved in inactivation of the *ER* gene in prostate cancer. Using the bisulfite genomic sequencing methods, we examined a 447-bp region of the *ER* promoter located immediately upstream from the transcribed sequence of the human *ER* gene.

MATERIALS AND METHODS

Cell Lines and Treatment. Human prostatic cancer cell lines LNCaP, PC3, and DU145 were obtained from American Type Culture Collection (Manassas, VA). ND1 (human primary prostate cancer) and BPH1 (human benign prostate epithelium) cell lines were developed in our laboratory (22, 23). DU145 and ND1 cells were cultured in DMEM. PC3 cells were cultured in F-12 Ham's. LNCaP, TSUPr1, DUPro, and BPH1 cells were maintained in RPMI 1640. All media were supplemented with 10% (v/v) fetal bovine serum and 2 mM L-glutamine. All media and supplements were obtained from the University of California–San Francisco Cell Culture Facility. For drug treatment, exponentially growing cells were seeded at a density of 2×10^6 cells/83 cm^2 flask (day 0). Cells were allowed to attach overnight before the addition of freshly prepared 5-azaC (Sigma Chemical Co., St. Louis, MO). On day 1, a final concentration of 2 μg/ml 5-azaC in PBS was added to the flask. The next day, the medium was changed. On day 3 and day 5, the cells were treated two more times as on day 1. On day 6, the cells were harvested.

Tissue Samples and Microdissection. Archival prostate cancer and BPH samples were obtained from the Veterans Affairs Medical Center in San Francisco and the University of California–San Francisco. Sections were cut 5 μm thick from formalin-fixed, paraffin-embedded tissues and mounted on microscope slide and diagnosed according to the Gleason scoring system. To collect cancer tissues for DNA extraction, microdissection was carried out as described previously (24).

Nucleic Acid Extraction. DNA from cell lines and microdissected tissues was extracted using QIAamp Tissue Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Total RNA was extracted by guanidium thiocyanate-phenol-chloroform extraction using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH).

Reverse Transcription-PCR. Total cellular RNA (1–5 μg) was reverse transcribed using random hexamers primer and Superscript II reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) in a 40-μl reaction.

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[1] Supported by NIH Grants DK-47517, CA-64872, AG-16870, and DK-52708; VA/DOD and VA REAP awards; and VA Merit Review (to R. D.).

[2] To whom requests for reprints should be addressed, at Urology Research Center (112F), 4150 Clement Street, San Francisco, CA 94121. Phone: (415) 750-6964; Fax: (415) 750-6639; E-mail: urolab@aol.com.

[3] The abbreviations used are: ER, estrogen receptor; DNA Mtase, DNA methlytransferase; BPH, benign prostate hyperplasia; 5-azaC, 5-aza-2′-deoxycytidine.

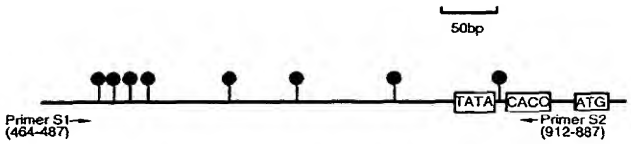

Fig. 1. Schematic representation of *ER* gene promoter structure. TATA, ATG signal, and CACC box are indicated. Primer S1 and primer S2 were used to amplify a 447-bp fragment for direct sequencing. The position of primers is indicated (GenBank accession number X68051). ●, CpG sites amplified by PCR.

cDNA was amplified by differential PCR using primers specific for the *ER* gene (GGAGACATGAGAGCTGCCA, sense; CCAGCAGCATGTCGAAGATC, antisense) and the *β-actin* gene (TCTACAATGAGCTGCGTGTG, sense; ATCTCCTTCTGCATCCTGTC, antisense). For amplification of *Dnmt 1* gene, primers TTCCATCCTTCTGCACAGG (sense) and TCTCCATCTTCGTCCTCGTCAG (antisense) were used, and the *β-actin* gene was also amplified as an interior control using primer TCTACAATGAGCTGCGTGTG (sense) and primer AATGTCAGGCACGATTTCCC (antisense). PCR reactions were performed in a PTC-200 thermal cycler (MJ Research, Watertown, MA) at 94°C for 1 min, 30 cycles at 94°C for 20 s, 57°C for 20 s, and 72°C for 30 s, followed by an extension step at 72°C for 5 min. The PCR products were electrophoresed through a 1.5% agarose gel containing ethidium bromide and were visualized by UV rays.

Bisulfite Genomic Sequencing. Bisulfite modification of genomic DNA was carried out according to reported methods (25). Modified DNA was amplified by two rounds of PCR with primers S1 (AAAGTGGTTAAGAGGTGGATTTA, upstream, sequence position 464 to 487; GenBank accession number X68051) and S2 (TCAAATTTACAAAATAAAACATAAA, downstream, sequence position 912 to 887; Fig. 1). The PCR conditions were 94°C

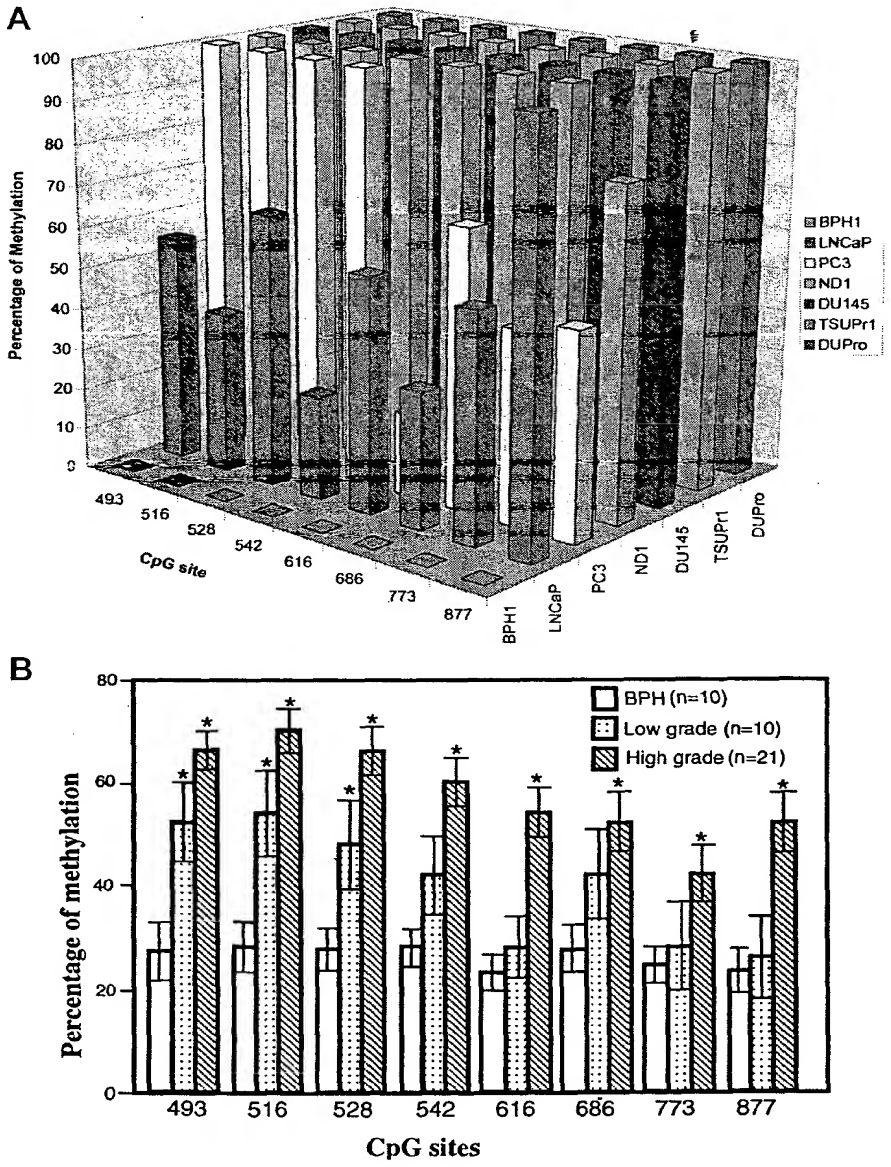

Fig. 2. ER methylation in prostate cell lines and tissue samples. DNA was bisulfite-modified and amplified by two rounds of PCR. The resulting PCR products were direct sequenced. *A*, *ER* methylation in prostate cell lines. Methylation percentage of the individual CpG site is presented as the ratio of methylated cytosine to the total cytosine (methylated plus unmethylated) in each cell line. *B*, *ER* methylation in prostate cancer and BPH tissues. The methylation percentage of the individual CpG site is presented as the average ratio of methylated cytosine to the total cytosine (methylated plus unmethylated) of all samples in each group. *, sites with significant difference in methylation between cancer and BPH samples. Numbers on *X* axis are the position of CpG sites (GenBank accession number X68051).

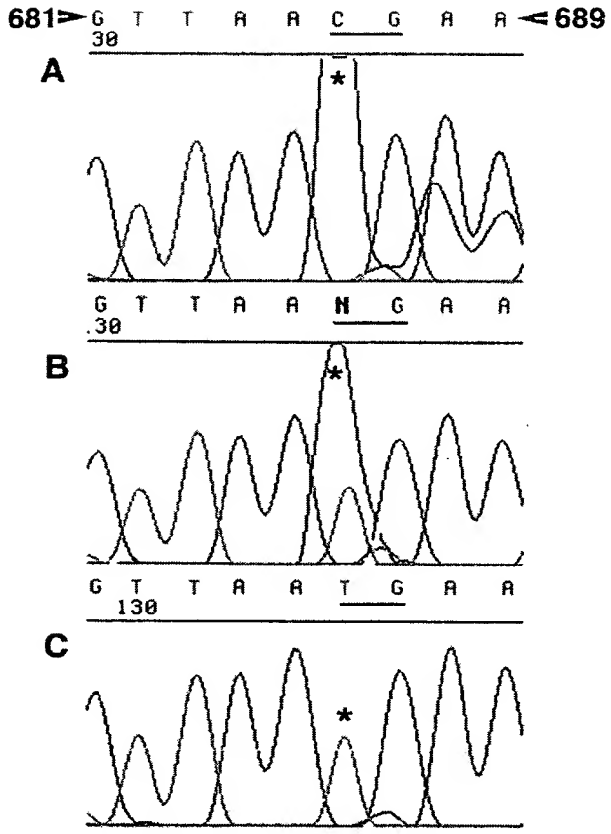

Fig. 3. Examples of direct sequencing chromatogram. DNA was first bisulfite-modified and PCR amplified. PCR products were then sequenced on an ABI automated sequencer with Dye terminators (Perkin-Elmer Corp.). Upper sequence represents GenBank sequence of the *ER* promoter (number X68051) from position 681–689. A CpG site is *underlined*, and the cytosine (*C*) or thymine (*T*) peaks are indicated by *asterisks*. *A*, complete methylation as found in prostate cancer cell lines. *B*, partial methylation as found in prostate cancer tissue. *C*, no methylation as found in BPH1 cell line.

for 2 min, 35 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. The first PCR products (1 μl) were subjected to a second round PCR cycled for 30 times. The resulting products were sequenced on an ABI automated sequencer with Dye terminators (Perkin-Elmer Corp., Foster City, CA).

Quantitation of Methylation Levels and Statistical Analysis. The methylation levels of individual CpG sites were expressed as percentages of 5-methyl cytosine among the whole cytosine population (methylated plus unmethylated) at the same site as shown on the computer-generated sequencing chromatogram (26). A single "C" at the corresponding CpG site was considered as 100% methylation, a single "T" as no methylation, and overlapping "C" and "T" as partial methylation. In the last case, the percentage of methylation was expressed as the ratio of "C" peak value to the peak values of "C" plus "T." Data are given as mean ± SE. Statistical analysis was performed using Student's *t* test for unpaired sample. Significance was defined as $P < 0.01$.

RESULTS

ER Methylation in Human Prostate Cancer Cell Lines. Using direct bisulfite genome sequencing techniques, we examined seven prostate cell lines (six cancerous and one normal) for *ER* gene promoter methylation. A 447-bp region (sequence position 464–912) of the proximal promoter of the *ER* gene, encompassing eight CpG sites, was amplified by PCR from bisulfite-modified DNA. The *ER* gene promoter structure is depicted in Fig. 1. Sequencing results revealed that all cytosines were converted to thymines, except those that existed as CpG doublet, and were methylated.

All of the six prostate cancer cell lines examined showed extensive methylation of the *ER* gene promoter (Fig. 2*A*). Of them, DU145, TSUPr1, DUPro, and ND1 exhibited 100% methylation at most of the CpG sites. PC3 and LNCaP were less methylated but still had sites that were 100% methylated. However, the normal cell line BPH1 did not show methylation at any CpG sites (Fig. 2*A*). We further treated *ER*-negative cell lines with the demethylating agent 5-azaC for 3 nonsuccessive days. After treatment, DNA was extracted and modified again, and the *ER* gene promoter was amplified using the same primers. Sequencing results of the PCR products from 5-azaC-treated cell lines revealed that most of the previously methylated CpG sites were demethylated completely with no methylated cytosine existing at the same CpG sites compared with that before treatment. Although the demethylation was not complete in some of the CpG sites, a lesser degree of methylation was observed when compared with the degree of methylation in nontreated cells (data not shown).

ER Methylation in Human Prostate Cancer Tissues. A total of 31 prostate cancer and 10 BPH tissue samples were analyzed for *ER* gene methylation. For cancer samples, we used microdissection techniques to collect tumor cells for DNA extraction and subsequent PCR amplification. Of the 31 prostate cancer tissue samples, 10 were low grade (including grades I and II) and 21 were high grade (including grades III-V). Overall, the percentages of samples that showed *ER* methylation were 60% (6 of 10) in BPH, 80% (8 of 10) in the low-grade cancer, and 96 (20 of 21) in the high-grade cancer. In contrast to methylation found in cell lines, no CpG site with complete methylation was found in tissue samples. To correlate methylation levels with tumor grades, we calculated the average methylation levels of individual CpG sites among each group. The resulting data from the cancer samples were compared with that from BPH samples using Student's *t* test. DNA from cancer exhibited a universally higher average of methylation at every CpG site than DNA from BPH. The differences between the high-grade samples and BPH samples were significant at every CpG site ($P < 0.01$; Fig. 2*B*). Only three sites (positions 493, 516, and 528) showed significantly higher methylation in the low-grade samples compared with the BPH samples ($P < 0.01$; Fig. 2*B*). Examples of sequencing chromatograms are displayed in Fig. 3.

Inactivation and Reactivation of the ER Gene in Prostate Cancer. To understand whether mRNA expression of *ER* is inactivated by *ER* promoter methylation, we performed RT-PCR analysis. No *ER* mRNA expression was found in all of the prostate cancer cell lines examined, whereas BPH1, the normal prostate epithelial cell line, showed normal levels of *ER* mRNA expression (Fig. 4*A*). The mRNA expression in cell lines correlated well with their methylation status, as revealed by sequencing study (Fig. 2*A*). Treatment of cell lines with 5-azaC for 3 nonsuccessive days restored *ER* mRNA expression in all

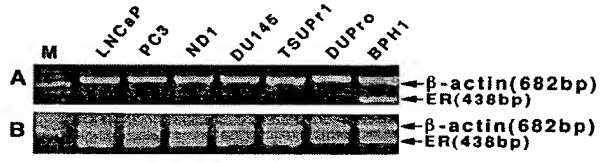

Fig. 4. ER mRNA expression in prostate cancer and BPH1 cell lines before (*A*) and after (*B*) demethylating agent treatment. Cells were treated for 3 nonsuccessive days with 5-azaC at a concentration of 2 μg/ml. After treatment, the cells were harvested for RNA extraction. Total cellular RNA (1–5 μg) was reverse transcribed, and the resulting cDNA was amplified with differential PCR using primers for the *β-actin* gene and *ER* gene.

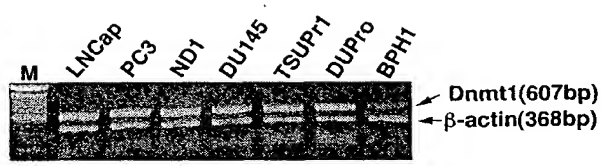

Fig. 5. mRNA expression of the *Dnmt1* gene in cell lines. Total cellular RNA (1–5 μg) was reverse transcribed, and the resulting cDNA was amplified with differential PCR using primers for the *β-actin* gene and *Dnmt1* gene.

of the cancer cell lines that showed no ER expression before treatment (Fig. 4*B*).

***Dnmt1* mRNA Expression** To understand the possible mechanism of *ER* methylation, we also examined the mRNA expression of the *Dnmt1* gene in the cell lines and found all of the cancer cell lines exhibited elevated levels of *Dnmt 1* expression, whereas, interestingly, BPH1 showed only minimal expression (Fig. 5).

DISCUSSION

The results of our study demonstrate that the *ER* gene is extensively methylated both in prostate cancer cell lines and prostate cancer tissues. The methylation levels correlate with tumor pathological grades and reversely correlate with *ER* gene expression. We also analyzed *ER* gene methylation in BPH tissues as a control. Surprisingly, the *ER* gene is also methylated in BPH tissue, although at much lower levels when compared with cancer. In human colorectal mucosa, physiological aging has been associated with *de novo* methylation in the *ER* gene (20). BPH is an age-related benign condition seen in the majority of men over 50 years and is characterized by stromal and/or glandular (epithelial) hyperplastic changes of the prostate. It is not clear whether ER methylation in BPH is an age-related event because data on ER methylation in normal prostate is lacking. Another possible explanation for *ER* methylation found in BPH is that it represents a disease-specific epigenetic mechanism that may contribute to pathogenesis of BPH. Although no link has been established between BPH and prostate cancer, it has been suggested that BPH and malignant prostate growth share a common origin because they commonly coexist and demonstrate androgen dependency (27). Therefore, BPH may be a premalignant lesion in prostate cancer development. The methylation of the *ER* gene in BPH could be one of the earliest events that predispose to prostate cancer.

There are two possible mechanisms of inactivation of the *ER* gene in prostate cancer. First, activators responsible for *ER* transcription are not available or transcriptional repressors predominate. Alternatively, the CpG island within the promoter region of the *ER* gene may be methylated, which leads to transcriptional inactivation of the *ER* gene through several undefined mechanisms. In the prostate cancer cell lines analyzed in our study, mRNA expression of the *ER* gene was well correlated with methylation status in those cell lines. Furthermore, *ER* mRNA expression was restored by treatment with demethylating agent 5-azaC, a cytosine analogue that acts as a suicide substrate for DNA methyltransferase when incorporated into DNA at the target site for DNA methylation, CpG dinucleotides (28). These results clearly indicate that the promoter methylation is the mechanism of *ER* inactivation in prostate cancer.

Promoter methylation is an epigenetic mechanism by which the gene is silenced. Unlike germline mutation, which may affect the whole cell population of the body, DNA methylation may be tissue-specific or cell-specific. For methylation analysis of tumor DNA, contamination by DNA from normal cells may present a major concern because different methylation patterns may exist in these two cell populations. In the present study, we isolated pure population of cancer cells using microdissection technique for methylation study. Our data should have reflected the methylation status of a pure cancer cell population rather than a mixture of malignant and nonmalignant cells.

In normal adult tissues, CpG islands are unmethylated, with the exception of transcriptionally silent genes on the inactive X chromosome and some imprinted genes (29, 30). An imbalance of DNA methylation, involving widespread hypomethylation, regional hypermethylation, and increased cellular capacity for methylation, is characteristic of human neoplasm. This imbalance begins in preneoplastic cells and becomes more extensive throughout subsequent stages of tumor progression (31). The data from our work showed a distinct trend of *ER* methylation in prostate cancer. With tumor progression, the *ER* is gradually methylated, leading to transcriptional inactivation. The most extensive methylation is observed in cultured cancer cell lines, which may be explained as the result of the fact that most of them were derived from metastatic prostate cancer.

The function of estrogen in the prostate has remained unclear. The hypothesis is proposed that estrogen interact with androgen in setting up the pace of prostatic growth and function. Estrogen not only directs stromal proliferation and secretion but also, through insulin-like growth factor I, conditions the response of the epithelium to androgen (32). The action of estrogen requires presence of its receptor, ER, in its target cells. The *ER* gene itself has metastasis suppressor properties in breast cancer cells (33) and suppresses the growth of many different cell types *in vitro* (34). Therefore, a tumor-suppressor role has been suggested (20). The altered methylation patterns observed during prostate cancer progression may possess wide implications in our understanding of the role of estrogen and its receptor in the pathogenesis and endocrinal manipulation of prostate cancer. Our study provides a clue that estrogen and its receptor may be involved in the initiation and progression of prostate cancer, as well as BPH.

To understand the potential mechanism of *ER* methylation, we examined in cell lines the mRNA expression of *Dnmt1*, the enzyme that methylates cytosines that are 5′ to guanosines and responsible for generating and maintaining DNA methylation patterns. *Dnmt1* expression was elevated in all of the cancer cell lines examined. Our results are consistent with the current concept that the level of Dnmt1 expression is elevated significantly in neoplastic cells compared with normal cells (33). Recent evidence showed that increased DNA Mtase activity is an early event in carcinogenesis (34). One possible molecular mechanism of this elevation of DNA Mtase in cancer cells is that the expression of the *Dnmt1* gene is regulated by oncogenic signaling pathways, such as the Ras-Jun signaling pathways (35, 36).

In conclusion, we demonstrate for the first time that *ER* gene methylation is a common event in prostate cancer, as well as BPH, can lead to inactivation of *ER* transcription, and is markedly associated with tumor progression. Our data offer insight into the mechanism by which *ER* is down-regulated in prostate cancer and may support the hypothesis that estrogen can have direct effects on prostate via its own receptor.

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Ciba Found Symp. 1983;96:230-41. Related Articles, Links

Antibodies to fetal antigens associated with rodent tumours.

Baldwin RW.

Fetal antigens associated with a range of carcinogen-induced and naturally arising rat tumours have been identified by reaction with antibodies induced by sensitizing rats for fetal cells in various ways, including by multiparity and by immunizing syngeneic WAB/Not rats with fetal tissues. Antibodies recognizing fetal antigens have potential applications in addition to their use for typing tumour-associated products. These applications include their use as carriers for targeting antitumour agents such as cytotoxic drugs and immunomodulating agents. Accordingly, several methods for producing antibodies directed against 'oncofetal' antigens have been examined, including the development of anti-fetal antibody-secreting hybridomas.

Publication Types:
- Review

PMID: 6343005 [PubMed - indexed for MEDLINE]

Toxicol Lett. 1995 Dec;82-83:335-40.

Related Articles, Links

A microassay for measuring cytosine DNA methyltransferase activity during tumor progression.

Belinsky SA, Nikula KJ, Baylin SB, Issa JP.

Inhalation Toxicology Research Institute, Albuquerque, NM 87185, USA.

The cytosine DNA methyltransferase (MT) enzyme, which catalyzes DNA methylation at CpG sites, is overexpressed at the mRNA level during the progressive stages of colon cancer. This paper describes the adaption of a sensitive microassay for determining MT enzyme activity during tumor progression in human colon and murine lung. MT activity was progressively elevated in mucosa from familial adenomatosis polyposis patients, mucosa adjacent to cancers, and in colonic adenocarcinomas when compared to colonic mucosa from control patients. In addition, the activity of this enzyme was increased in alveolar type II but not Clara cells isolated from A/J mice following carcinogen exposure and continued to increase during tumor progression. The use of a microassay for measuring MT activity indicates that changes in enzyme activity were in general agreement with previous findings of increased MT mRNA levels during colon cancer progression and also implicates the involvement of this pathway in lung cancer development.

PMID: 8597074 [PubMed - indexed for MEDLINE]

Proc Natl Acad Sci U S A. 1996 Apr 30;93(9):4045-50.

Increased cytosine DNA-methyltransferase activity is target-cell-specific and an early event in lung cancer.

Belinsky SA, Nikula KJ, Baylin SB, Issa JP.

Inhalation Toxicology Research Institute, Albuquerque, NM 87185, USA.

The association between increased DNA-methyltransferase (DNA-MTase) activity and tumor development suggest a fundamental role for this enzyme in the initiation and progression of cancer. A true functional role for DNA-MTase in the neoplastic process would be further substantiated if the target cells affected by the initiating carcinogen exhibit changes in enzyme activity. This hypothesis was addressed by examining DNA-MTase activity in alveolar type II (target) and Clara (nontarget) cells from A/J and C3H mice that exhibit high and low susceptibility, respectively, for lung tumor formation. Increased DNA-MTase activity was found only in the target alveolar type II cells of the susceptible A/J mouse and caused a marked increase in overall DNA methylation in these cells. Both DNA-MTase and DNA methylation changes were detected 7 days after carcinogen exposure and, thus, were early events in neoplastic evolution. Increased gene expression was also detected by RNA in situ hybridization in hypertrophic alveolar type II cells of carcinogen-treated A/J mice, indicating that elevated levels of expression may be a biomarker for premalignancy. Enzyme activity increased incrementally during lung cancer progression and coincided with increased expression of the DNA-MTase activity are strongly associated with neoplastic development and constitute a key step in carcinogenesis. The detection of premalignant lung disease through increased DNA-MTase expression and the possibility of blocking the deleterious effects of this change with specific inhibitors will offer new intervention strategies for lung cancer.

PMID: 8633014 [PubMed - indexed for MEDLINE]

Med Hypotheses. 1994 Aug;43(2):119-23. Related Articles, Links

On the oncodevelopmental role of human imprinted genes.

Biran H, Ariel I, De Groot N, Hochberg A.

Department of Biochemistry, Hebrew University, Jerusalem, Israel.

Genome imprinting has an essential role in normal embryonal mammalian development. Starting early in differentiation, the transcripts of certain human genes, e.g. the paternally-H19 and the maternally-imprinted IGF2, are expressed in specific tissues and organs during fetal life. In several malignant disorders, imprinted genes are, again, unfolded. Characteristically, expression follows the same tissue presentation as during embryogenesis. Clinical paternal disomies, i.e. trophoblastic diseases, and their maternal counterpart, i.e. ovarian teratomas, are associated with apparent relaxation of imprinting once they turn malignant. Paediatric neoplasms, like Wilm's tumor (WT) and rhabdomyosarcoma, often express IGF2 and H19. Recently, we have found H19 expression in invasive urothelial cancer. Evidently, imprinted genes display an oncodevelopmental mode of expression, very much like the classical oncofetal proteins AFP and CEA. Based on available data, including tumor preferential paternal allele retention and chromosome 11 short arm physical linkage with oncogenes like H-ras, we hypothesize that imprinted genes not only accompany cancer but may play a causative role as well.

Publication Types:
- Review
- Review, Tutorial

PMID: 7527480 [PubMed - indexed for MEDLINE]

Ciba Found Symp. 1983;96:28-54. Related Articles, Links

Embryonic antigens in malignancy and pregnancy: common denominators in immune regulation.

Coggin JH Jr.

There is mounting that the majority of malignant tumours of humans and rodents carry embryonic determinants (EA) associated with the cell membrane. The evolutionary and developmental purpose of these determinants seems to be related to their biochemical and structural role in the cell membrane. True EAs are uniquely expressed on germinal, embryonic and some fetal cell membranes and are not expressed in adult tissues nor in regenerating tissues. The immunological role of EA in fetal development in utero is still obscure. It is known that maternal IgG and cytotoxic T lymphocytes are produced in response to EA expressed in utero. Immunoregulatory interactions between suppressor, helper and cytotoxic T lymphocytes sensitized to EA during pregnancy have been detected in mice and hamsters bearing a variety of tumours. The same immunological events may occur in humans. The biological product of the retrogenic process that leads to the expression of EA in the emerging malignant cell clone seems to be intimately associated with the promotion of tumour-protective immune responses in the host which mimic the immune responses in pregnancy aimed at protecting the EA+ fetus from maternal immune attack. The description of the immunoregulatory perturbations generated by EA during the induction of cancer may eventually provide effective and predictable attack points for immunological intervention in the control of malignancy. This paper describes methods for detecting EA and considers some immunoregulatory traits found in response to EA expression.

Publication Types:
- Review

PMID: 6189676 [PubMed - indexed for MEDLINE]

Immunol Lett. 2000 Sep 15;74(1):27-34. Related Articles, Links

ELSEVIER SCIENCE
FULL-TEXT ARTICLE

Antitumor vaccination using peptide based vaccines.

Eisenbach L, Bar-Haim E, El-Shami K.

Department of Immunology, The Weizmann Institute of Science, 76100, Rehovot, Israel. lea.eisenbach@wezmann.ac.il

Cytotoxic T Lymphocytes (CTLs) recognize Major Histocompatibility Complex (MHC) class I in complex with peptides. Peptides derived from Tumor Associated Antigens (TAAs) are therefore targets for tumor rejection. A number of TAAs were identified in the last decade from human and murine tumors. Here we summarize the methods for TAA and peptide identification, the nature of TAA peptides and the making of antitumor vaccines.

Publication Types:
- Review
- Review, Tutorial

PMID: 10996624 [PubMed - indexed for MEDLINE]

Proc Natl Acad Sci U S A. 1991 Apr 15;88(8):3470-4.

High expression of the DNA methyltransferase gene characterizes human neoplastic cells and progression stages of colon cancer.

el-Deiry WS, Nelkin BD, Celano P, Yen RW, Falco JP, Hamilton SR, Baylin SB.

Oncology Center, Baltimore, MD 21231.

DNA methylation abnormalities occur consistently in human neoplasia including widespread hypomethylation and more recently recognized local increases in DNA methylation that hold potential for gene inactivation events. To study this imbalance further, we have cloned and localized to chromosome 19 a portion of the human DNA methyltransferase gene that codes for the enzyme catalyzing DNA methylation. Expression of this gene is low in normal human cells, significantly increased (30- to 50-fold by PCR analysis) in virally transformed cells, and strikingly elevated in human cancer cells (several hundredfold). In comparison to colon mucosa from patients without neoplasia, median levels of DNA methyltransferase transcripts are 15-fold increased in histologically normal mucosa from patients with cancers or the benign polyps that can precede cancers, 60-fold increased in the premalignant polyps, and greater than 200-fold increased in the cancers. Thus, increases in DNA methyltransferase gene expression precede development of colonic neoplasia and continue during progression of colonic neoplasms. These increases may play a role in the genetic instability of cancer and mark early events in cell transformation.

PMID: 2014266 [PubMed - indexed for MEDLINE]

Oncology. 2001;60(1):1-7. Related Articles, Links

KARGER Full Text

Vaccination for malignant melanoma: recent developments.

Jager D, Jager E, Knuth A.

II. Medizinische Klinik, Hamatologie-Onkologie, Krankenhaus Nordwest, Frankfurt am Main, Deutschland.

The identification of tumor-associated antigens recognized by cellular or humoral effectors of the immune system has opened new perspectives for cancer immunotherapy. Different categories of cancer-associated antigens have been described as targets for CD8+ T cells in vitro and in vivo: (1) 'cancer-testis' (CT) antigens expressed in different tumors and normal testis; (2) melanocyte differentiation antigens; (3) point mutations of normal genes; (4) antigens that are overexpressed in malignant tissues, and (5) viral antigens. Clinical trials with antigenic peptides have been initiated to induce specific immunological responses in vivo. Immunological and clinical parameters for the assessment of peptide-specific reactions have been defined: DTH, CD8+ T cell, autoimmune and tumor regression responses. Preliminary results show that tumor-associated peptides alone elicit specific DTH and CD8+ T cell responses associated with tumor regression after intradermal vaccination. Granulocyte macrophage colony-stimulating factor has been shown to enhance peptide-specific immune reactions by amplification of dermal antigen-presenting dendritic cells. Complete tumor regressions have been observed after the induction of CD8+ T cell responses by peptide immunization. Based on these results, active immunotherapy with tumor-associated antigens may be a promising approach for patients in adjuvant treatment situations, who are at high risk for tumor recurrence. Recently, a strategy utilizing spontaneous antibody responses to tumor-associated antigens (SEREX) has led to the identification of a new CT antigen, NY-ESO-1. NY-ESO-1-specific spontaneous humoral and cellular immune responses were found in approximately 50% of patients with NY-ESO-1-positive tumors. Clinical studies have been initiated to evaluate the immunological effects of immunization with NY-ESO-1 peptides in cancer patients with detectable or absent immunity against NY-ESO-1.

Publication Types:
- Review
- Review, Tutorial

PMID: 11150901 [PubMed - indexed for MEDLINE]

Clin Breast Cancer. 2001 Apr;2(1):73-9.

Expansion of HER2/neu-specific T cells ex vivo following immunization with a HER2/neu peptide-based vaccine.

Knutson KL, Disis ML.

Division of Oncology, University of Washington, 1959 NE Pacific Street, HSB BB1321, Box 356527, Seattle, WA 98195-6527, USA. kknutson@u.washington.edu

The identification and characterization of tumor antigens has facilitated the development of immune-based cancer prophylaxis and therapy. Cancer vaccines, like viral vaccines, may be effective in cancer prevention. Adoptive T-cell therapy, in contrast, may be more efficacious for the eradication of existing malignancies. Our group is examining the feasibility of antigen-specific adoptive T-cell therapy for the treatment of established cancer in the HER2/neu model. Transgenic mice overexpressing rat neu in mammary tissue develop malignancy, histologically similar to human HER2/neu-overexpressing breast cancer. These mice can be effectively immunized against a challenge with neu-positive tumor cells. Adoptive transfer of neu-specific T cells into tumor-bearing mice eradicates malignancy. Effective T-cell therapy relies on optimization of the ex vivo expansion of antigen-specific T cells. Two important elements of ex vivo antigen-specific T-cell growth that have been identified are (1) the preexisting levels of antigen-specific T cells and (2) the cytokine milieu used during ex vivo expansion of the T cells. Phase I clinical trials of HER2/neu-based peptide vaccination in human cancer patients have demonstrated that increased levels of HER2/neu-specific T-cells can be elicited after active immunization. Initiating cultures with greater numbers of antigen-specific T cells facilitates expansion. In addition, cytokines, such as interleukin-12, when added during ex vivo culturing along with interleukin-2 can selectively expand antigen-specific T-cells. Interleukin-12 also enhances antigen-specific functional measurements such as interferon-gamma and tumor necrosis factor-alpha release. Refinements in ex vivo expansion techniques may greatly improve the feasibility of tumor-antigen T-cell-based therapy for the treatment of advanced-stage HER2/neu-overexpressing breast malignancy.

Publication Types:
- Review
- Review, Tutorial

PMID: 11899386 [PubMed - indexed for MEDLINE]

Anticancer Res. 1998 Jul-Aug;18(4A):2575-82. Related Articles, Links

Examination of the DNA methylation properties in nontumorigenic and tumorigenic breast epithelial cell lines.

Pilat MJ, Schwab ED, Yao KL, Pienta KJ.

University of Michigan, Department of Internal Medicine, Ann Arbor 48109-0946, USA.

Molecular changes in the progressive state of tumorigenesis often include altered patterns of DNA methylation. Utilizing a series of breast epithelial cell lines, the overall 5-methylcytosine content in genomic DNA demonstrated an overall decrease when comparing two malignant cell lines (MCF-7 and T47D) with a mortal cell line (MCF 1 2M) and several derivative cell lines of the immortalized MCF10 cultures (MCF10A,-2A, -5A, A1neoT2, and 139B6). Further investigation on the methylation status of these cells lines indicated no difference in DNA methyltransferase activity, both at a protein and mRNA levels, in the nontumorigenic cell lines examined while activity was 3-10 fold higher in the tumorigenic lines (MCF7, T47D, SkBr3, MB-MDA-231, -468). Examination of the CpG island in the 5' promoter region of the estrogen receptor gene indicates that this region is unmethylated in the mortal and immortal nontumorigenic lines as well as the tumorigenic lines examined, with the exception of the estrogen receptor negative breast cell line MB-MDA-468 which appears to be partially methylated at this site. These results indicate methylation of this CpG island does not account for the inactivation of the estrogen receptor gene in immortalized nontumorigenic breast cells, suggesting another mechanism of transcriptional inactivation of ER in this environment.

PMID: 9703912 [PubMed - indexed for MEDLINE]

Int J Oncol. 2000 Jan;16(1):81-96. Related Articles, Links

Vaccination against human cancers (review).

Sinkovics JG, Horvath JC.

Cancer Institute, St. Joseph's Hospital, Tampa, FL 33607, USA.

Classical and molecular immunological means of active tumor-specific immunization against human cancers yielded whole cell or tumor cell lysate vaccines of preventive value (reduced relapse rates) and dendritic cell-peptide or genetically engineered vaccines that may induce remissions even in metastatic disease. Active tumor-specific immunization was often successful in the past 50 years against experimental tumors maintained in the laboratory. During the epochs of classical and molecular immunology several vaccines were generated and used for the reduction of relapse rates of human cancer after surgical removal of the primary or metastatic tumors. Whole cell vaccines consist of X-irradiated autologous or allogeneic tumor cells coadministered with immunostimulants (BCG, Detox). Tumor cells haptenized biologically (as in viral oncolysates) or chemically were also used. Dendritic cell vaccines are prepared by transfection or transduction with tumor antigen-encoding DNA or by pulsing the cells with antigenic peptides in vitro; or collecting dendritic cells that engulfed apoptotic tumor cell DNA and/or peptide antigens in vivo for reinjection into the patient. Genetically engineered tumor cells are prepared in vitro to express MHC and peptides, costimulatory molecules (B7.1) and cyto- or lymphokines (interferons, interleukins, hematopoietic growth factors) for vaccination of patients. Antibody- and immune T cell-mediated immune reactions to autologous tumor cells are newly generated and/or quantitatively increased in immunized patients but do not always correlate with clinical response. Most vaccines are claimed to have reduced relapse rates presumably by inducing effective host immunity against micrometastases. Dendritic cell-peptide vaccines could induce partial or occasionally complete remissions in metastatic disease. The wrong antigenic presentation may result in tolerance induction toward the tumor; occasionally tumor enhancement may occur. Human tumor antigens when presented appropriately (with costimulatory molecules and with IL-2, IL-12) break the host's natural tolerance toward its tumor and induce rejection strength immune reactions even in patients with metastatic disease. Immune T cells thus generated could be collected for adoptive immunotherapy. For successful active specific immunization against human cancers the understanding of the immunoevasive maneuvers of the tumor cell (through FasL --> Fas; TRAIL; CD40L --> CD40; TGFbeta etc. systems) is essential.

Publication Types:
- Review
- Review, Academic

PMID: 10601552 [PubMed - indexed for MEDLINE]

Pharmacol Ther. 1996;70(1):1-37. Related Articles, Links

ELSEVIER SCIENCE
FULL-TEXT ARTICLE

The DNA methylation machinery as a target for anticancer therapy.

Szyf M.

Department of Pharmacology and Therapeutics, McGill University, Montreal, Canada.

DNA methylation is now recognized as an important mechanism regulating different functions of the genome; gene expression, replication, and cancer. Different factors control the formation and maintenance of DNA methylation patterns. The level of activity of DNA methyltransferase (MeTase) is one factor. Recent data suggest that some oncogenic pathways can induce DNA MeTase expression, that DNA MeTase activity is elevated in cancer, and that inhibition of DNA MeTase can reverse the transformed state. What are the pharmacological consequences of our current understanding of DNA methylation patterns formation? This review will discuss the possibility that DNA MeTase inhibitors can serve as important pharmacological and therapeutic tools in cancer and other genetic diseases.

Publication Types:
- Review
- Review, Academic

PMID: 8804109 [PubMed - indexed for MEDLINE]

Cancer Metastasis Rev. 1998 Jun;17(2):219-31. Related Articles, Links

Targeting DNA methyltransferase in cancer.

Szyf M.

Department of Pharmacology, McGill University, Montreal, PQ, Canada.
mszyf@pharma.mcgill.ca

DNA methyltransferase is an enzyme responsible for generating and maintaining DNA methylation patterns. DNA methylation patterns control different genome functions, thus they are an important component of the epigenetic information. It has been recently postulated that DNA methyltransferase plays an important role in oncogenesis and that it is a candidate target for anticancer therapy. This commentary discusses the possible mechanisms through which DNA methyltransferase participates in oncogenesis and the rationale for targeting it in cancer.

Publication Types:
- Review
- Review, Tutorial

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Synthetic immunogens, containing built-in adjuvanticity, B cell, T helper cell and CTL epitopes or mimotopes, are ideal and invaluable tools to study the immune response with respect to antigen processing and presentation. This serves as a basis for the development of complete and minimal vaccines which do not need large carrier proteins, further adjuvants, liposome formulations or other delivery systems. Combinatorial peptide libraries, either completely random or characterized by one or several defined positions, are useful tools for the identification of the critical features of B cell epitopes and of MHC class I and class II binding natural and synthetic epitopes. The complete activity pattern of an O/Xn library with hundreds of peptide collections, each made up from billions of different peptides, represents the ranking of amino acid residues mediating contact to the target proteins of the immune system. Combinatorial libraries support the design of peptides applicable in vaccination against infectious agents as well as therapeutic tumour vaccines. Using the principle of lipopeptide vaccines, strong humoral and cellular immune responses could be elicited. The lipopeptide vaccines are heat-stable, non-toxic, fully biodegradable and can be prepared on the basis of minimized epitopes by modern methods of multiple peptide synthesis. The lipopeptides activate the antigen-presenting macrophages and B cells and have been recently shown to stimulate innate immunity by specific interaction with receptors of the Toll family.

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TITEL

A NOVEL DNA METHYL TRANSFERASE I-DERIVED PEPTIDE ELUTED FROM SOLUBLE HLA-A*0201 INDUCES PEPTIDE-SPECIFIC TUMOR DIRECTED CYTOTOXIC T CELLS

AUTHORS

Martina Berg[1], Eilon Barnea[2], Arie Admon[2] and Nicholas Zavazava[1]

Affiliation:

[1]Department of Internal Medicine, University of Iowa Hospital and Clinics & VA Medical Center, Iowa City; IA, USA

[2]The Smoler Protein Center, Department of Biology, Technion, Haifa, Israel

CORRESPONDING AUTHOR

Nicholas Zavazava

University of Iowa Hospitals and Clinics & VA Medical Center Iowa City

Department of Internal Medicine, C51-F

200 Hawkins Drive

Iowa City, IA 52242, USA

Phone (319) 384 6577

Fax (319) 356 8280

Email nicholas-zavazava@uiowa.edu

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ABBREVIATIONS

MHC	major histocompatibility complex
HLA	human leukocyte antigen
DNMT	DNA methyl transferase
LMP	low molecular weight protein
TAP	transporter associated with antigen processing
CTL	cytotoxic T lymphocytes
IFN	interferon
DC	dendritic cells
TAA	tumor associated antigens
APC	antigen presenting cells
TcR	T cell receptor
NK	natural killer cell

ABSTRACT

Mutations in the genes involved in antigen processing and presentation are some of the strategies by which tumors evade recognition by cytotoxic T cells (CTL). Recently, we identified novel HLA-A*0201 (HLA-A2) restricted peptides recovered from soluble HLA molecules secreted by human tumor cell lines, transfected with truncated genes of HLA-A2 and HLA-B7. Here, four candidate peptides eluted from soluble HLA-A2 were selected on the basis of their precursor proteins that are tumor-associated antigens (TAA). Peptide p1028 (GLIEKNIEL), derived from DNA methyl transferase I (DNMT-1), which is over-expressed in the majority of human tumors, showed the highest affinity to HLA-A2 and was most abundant in the sMHC/peptide complexes of all transfected breast, ovarian and prostate cancer cell lines. Peptide p1028 specific CTL were generated in vitro and shown not only to efficiently lyse target cells pulsed with the peptide, but also the HLA-A2 positive breast cancer cell lines MDA-231 and MCF-7. The peptide induced IFN-γ production in the CTL, which were selectively stained by a p1028-tetramer. Since DNMT-1 is a widely expressed tumor-associated enzyme, the novel DNMT-1-derived HLA-A2 restricted peptide GLIEKNIEL identified here, may provide a suitable candidate for a broadly applicable therapeutic cancer vaccine.

INTRODUCTION

The ideal tool for the elimination of tumor cells should utilize cytotoxic T cells (CTL), because of their specificity for peptides (1-4). One obstacle for the successful application of T cell based immunotherapy is that tumors have evolved a number of different strategies to escape recognition by T cells. Since CTL recognize tumor antigens in the context of HLA class I molecules, alteration of HLA surface expression is a major mechanism used by tumors to escape immune surveillance (5). Lack of HLA expression can be induced by any mutations affecting HLA synthesis, assembly, transport, or expression on the cell surface (6). Thus, each step of the antigen processing and presentation pathway can be a target for escape of recognition by T cells. For example, deficiencies of proteasome components, as the subunits LMP2 and LMP7, and of TAP molecules as well as β2-microglobulin downregulation have been observed in various human tumors and could lead to an impairment in the processing of tumor associated antigens (TAA) and in their presentation as antigenic peptides to CD8+ T cells, as recently reviewed by Seliger (7).

However, despite those escape mechanisms, human clinical trials for a variety of malignant diseases using T cell therapy proved to be highly effective and curative for some individuals (8). Recently, a number of TAA have been identified, mainly melanoma antigens, such as Melan-A and MART-1. Studies were initiated to vaccinate tumor patients against these antigens and their ability to induce anti-tumor T cell immunity has been shown in clinical studies (9). Many of the recently identified tumor antigens are non-mutated differentiation antigens overexpressed in tumors, e.g. a peptide derived from protein tyrosinase, which was shown to successfully induce the attack and lysis of

melanoma cells from cancer patients (10). Several strategies have been evolved to identify novel tumor associated antigens, e.g. the cloning of epitopes recognized by CTL (11) and the SEREX technology (12), where patients antibodies are used to identify tumor antigens from a cDNA expression library made from mRNA of tumor specimens. Conventionally, endogenous peptides have been eluted from affinity purified HLA followed by analysis using HPLC, gas spectrometry and sequencing (13, 14). The purification of the MHC molecules is complicated due to unavoidable contamination of MHC molecules with cell debris and detergents after cell lysis. We recently identified HLA-restricted tumor-specific antigens presented by the soluble MHC class I molecule by transfecting human tumor cell lines with truncated genes of HLA-A2 after purification of the secreted soluble HLA by affinity chromatography (15). Novel peptides unique to the soluble MHC variants were identified. The discovered peptide-pool was screened for peptides derived from tumor-relevant antigens, which were most highly expressed in the soluble MHC. Our hypothesis is, that tumors redirect tumor relevant peptides into the soluble form of the MHC instead of displaying them on the cell surface in the membrane bound MHC class I molecule.

HLA class I molecules are integral membrane glycoproteins, however, the presence of a soluble form of these molecules was demonstrated decades ago (16, 17). Three different forms of soluble MHC were identified in human plasma: the 44 kDa intact heavy chain, which is produced by membrane shedding, a 39 kDa variant which is due to alternate splicing of mRNA and a 36 kDa protein, which is truncated and lacks both transmembrane domain and cytoplasmic tail, believed to be a product of proteolysis by metalloproteinases (18, 19). As with membrane bound MHC, soluble MHC class I

molecules bind to peptides which are 8 to 10 aa long, with a few exceptions having a length of 6, 11 and 12 aa (15). Up to date, there are only a few reports regarding the amount of secreted MHC class I by tumor cells *in vivo*. The expression of sHLA seems to be dependent on the type and stage of the tumor, resulting in non-consistent reports regarding the amount of secreted sHLA detected in plasma of cancer patients (20, 21).

Here, we selected four peptides from the pool of soluble MHC derived peptides identified on the basis of their relative abundance and relevance to tumors. Although peptides have been eluted and sequenced from a membrane-bound HLA-A*0201 by several groups, none of these peptides have been so far identified. Thus, these novel peptides eluted from the truncated HLA-A*0201 provide interesting candidates for tumor vaccines.

MATERIALS AND METHODS

Cell culture

Breast cancer cell lines MCF-7 and MDA-231, and the TAP-transporter deficient lymphoblastic T2 cell line were maintained at 37 °C in a 5 % CO_2 incubator in RPMI plus HEPES (Gibco, Grand Islands, NY, USA) supplemented with 100 U/ml streptomycin/penicillin (Gibco). All cell lines were purchased from American Type Culture Collection (Rockville, MD, USA).

Western Blotting

$5x10^6$ MCF-7 cells were extracted in lysis buffer containing 1 % NP-40, 0.15 M NaCl, and 1M Tris pH 7.4. As control supernatants, we used genetically engineered recombinant soluble HLA-A2 (sA2) and soluble HLA-A2 with a Bsp-tag (sA2-Bsp). The protein samples were denatured in reducing sample buffer, electrophoresed on 10 % SDS-polyacrylamide gels and transferred to nitrocellulose for 4 h at 0.8 Amp.

The transfer membrane was blocked with 5 % (w/v) bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) in PBS and incubated with a rabbit anti-human MHC class I heavy chain antiserum, kindly provided by Dr. Stam (Amsterdam, Netherlands), or with the 4B5 anti-HLA-A2 monoclonal antibody, raised in our laboratory. This antibody was generated by immunizing mice with denatured HLA-A2 and HLA-B7. It preferentially binds the 36 kDa MHC variant. The membrane was incubated with a goat-anti-rabbit or goat-anti-mouse peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology,

Santa Cruz, CA, USA), respectively, and developed by using ECL western blot detection reagents (Amersham, Piscataway, NJ, USA).

Peptide-binding assay

Each peptide was tested for binding to T2 cells in an HLA-A2 stabilization assay (22). Briefly, TAP deficient T2 hybridoma cells were incubated with 25μg/ml peptide and 15μg/ml β2-microglobulin (Research Diagnostics, Flanders, NJ, USA) at 37°C for 24 h. Expression of HLA-A2 was assayed by flow cytometry after staining the cells with the anti-HLA-A2 Ab PA2.1 (ATCC, Rockville, MD, USA) and FITC-conjugated $F(ab')_2$ goat anti-mouse Ig (BD Pharmingen, San Diego, CA, USA).

Preparation of HLA-A*0201 positive PBMC and dendritic cells

PBMCs from healthy donors were purified by centrifugation in Ficoll-Hypaque (Sigma, St. Louis, MO, USA) from leukapheresis products obtained from healthy volunteers. For *in vitro* generation of peptide-specific CTLs, we generated autologous DCs as APCs. DCs were generated from fresh PBMCs in 2 steps, as previously described (23). Briefly, monocyte-enriched cell fractions were prepared by 2 h plastic adherence of 6.67×10^6 PBMCs/ml and 0.2 ml/cm^2 and cultured for 7 days in X-VIVO-15 medium (Biowhittaker, Walkersville, MD, USA) supplemented with 1% autologous plasma, 800 U/ml GM-CSF and 500 U/ml IL-4. On day 7, the non-adherent cell population was harvested; resuspended in fresh culture medium containing 800 U/ml GM-CSF, 500 U/ml IL-4, 1000 U/ml IL-6, 10 ng/ml IL-1 and 10 ng/ml TNF-α and transferred to fresh tissue

culture flasks (all cytokines were purchased from PeproTech, Rocky Hill, NJ, USA). On day 10, non-adherent cells were harvested and screened for typical cell-surface markers.

Flow Cytometry

For flow cytometry, 2-5x10^5 mature and immature DCs were incubated for 30 minutes in the dark at 4 °C with FITC-labeled anti-CD80, -CD83, -CD86, -CD11c and PE labeled anti HLA-DR antibodies (all antibodies were purchased from BD Pharmingen). Stained cells were detected with a FACScan (Becton Dickinson) and analyzed with the WinMDI software.

Purification of $CD8^+$ T cells

CD8 T cells were enriched using MACS Micro beads (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's directions. Monoclonal antibodies for CD4 and CD8 (BD Pharmingen) were used to monitor cell purity by flow cytometry.

Induction of CTL

DCs were pulsed for 4 h at 37 °C with 50 μg/ml peptide p1028 in the presence of 3 μg/ml β2-microglobulin (Research Diagnostics, Flanders, NJ, USA) and inactivated by irradiation before use. $CD8^+$ T lymphocytes were co-cultured at 2x10^6 cells/well with 6.7x 10^5 peptide-pulsed DCs/well in 24-well plates in 2ml RPMI-1640 supplemented with 10% autologous plasma, 25 mM HEPES, 0.05 mg/ml gentamicin, 20 U/ml IL-2 and 5 U/ml IL-4 (PeproTech). On day 12 and weekly thereafter, CTL were restimulated with peptide-pulsed DCs as described above.

Cytotoxicity assay

Lysis of target cells by CTL recognizing peptide p1028 was tested in a standard 4 h-^{51}Cr-release assay. Briefly, $1.25x10^5$ target cells (T2 cells pulsed or unpulsed, MCF-7, MDA-231, DC) were labeled for 1 h at 37 °C with 180 µCi of $Na_2{}^{51}CrO_4$ (PerkinElmer, Boston, MA, USA), washed three times, resuspended in RPMI/10% FCS and plated in conical 96-well plates. CTL were added to target cells at indicated E/T ratios to a total volume of 200 µl/well. After 4 h at 37 °C, the supernatants were harvested by using harvesting frames and detected in a gamma counter. Percent specific lysis was calculated as (cpm experimental counts – cpm media control) / (cpm TritonX-100 – cpm media control) x 100%.

Spontaneous release was determined from the wells to which 100µl of medium were added instead of effector cells. Total releasable radioactivity was obtained after treating the target cells with 1 % Triton X-100.

Intracellular IFN-γ assay

The specificity of the CTL for p1028 was further tested by measuring intracellular IFN-γ release. $1x10^5$ CTL were co-incubated with $1x10^3$ peptide-pulsed T2 cells in 96-well plates in a volume of 100 µl/well. Control wells contained CTL alone or CTL in the presence of unloaded T2 cells. Intracellular IFN-γ was measured after 24 h incubation at 37 °C. Cells were stained with anti-CD8-FITC for 30 min at 4 °C. After permeabilization, following the manufacturers protocol, they were incubated with PE-conjugated IFN-γ -

antibody (antibodies and Cytofix/Cytoperm Kit was purchased from BD Pharmingen). Cell fluorescence was analyzed by flow cytometry.

Tetramer Staining

To further determine the specificity of the CTL, tetramers were generated. These tetramers were produced by ProImmune, Oxford, UK. 2-5x10^5 CTL were washed with PBS and resuspended in 50 µl PBS/1%BSA. The cells were incubated for 15 min on ice with 1 µg/ml FITC-labeled tetramer, while PE conjugated anti-CD8 Ab (BD Pharmingen) was added for 20 min. After washing, stained cells were detected with a FACScan.

RESULTS

Production of soluble HLA-A2 by breast cancer cell lines MDA-231 and MCF-7

MDA-231 and MCF-7 are two of the tumor cell lines previously used for the identification and purification of soluble HLA-A2-derived peptides after transfection with the truncated HLA-A2 gene (15). Sufficient amounts of sHLA-A2/peptide complexes were secreted by $5x10^7$ cells to analyze the recovered peptides. Here, we investigated the production of soluble MHC class I variants by the parental, non transfected tumor cell lines. Three different variants with molecular weights of 36, 39 and 44 kDa are produced by MDA-231, as detected in the cell lysate, Figure 1*a*. The 36 kDa protein band matches that of sA2 and sA2-Bsp. However, the two breast cancer cell lines did not release detectable amounts of soluble MHC into the supernatants, whereas strong expression of the truncated 36 kDa heavy chain was detected in both cell lysates, Figure 1*b*. Supernatant containing recombinant soluble HLA-A2, produced by transfected cell lines, was used as control. Staining was performed with the rabbit-anti-human polyclonal antibody. These results indicate that those tumor cell lines produce soluble forms of the MHC, particularly high amounts of the truncated 36 kDa protein, clearly retaining this protein in the cytosol, without releasing it into the supernatant. Interestingly, the 36 kDa MHC variant is a product of enzymatic proteolysis.

Selection of soluble HLA-A2-derived peptides

We selected the most abundant peptides eluted from sA2, whose precursor proteins were tumor-associated, and screened those for their binding affinity to HLA-A2. The sMHC

derived peptides characterized here are 8 to 10 amino acids long with one exception, peptide p1258 (Table 1), which has a length of 11 amino acids. The binding affinities of these peptides to HLA-A2 were tested using T2 cells that are TAP deficient. The low level of peptides in this TAP-mutant T2 cells causes most MHC class I molecules to remain empty or to associate with high and low affinity peptides. Upon exposure to exogenous HLA-A2-binding peptides enhanced HLA-A2 expression can be observed and detected by flow cytometry, Figure 2. Peptide p1028 (GLIEKNIEL) has the highest binding affinity to HLA-A*0201 compared to the other three peptides. It displays with 9 amino acids the length of a typical MHC class I ligand and shows the characteristic anchor residues with leucine at the second and the last amino acid.

The finding of relatively high affinity for p1028 is consistent with the theoretical score in the database for MHC ligands and peptide motifs SYFPEITHI (24), where this epitope is scored with 28, whereas the maximal theoretical score for HLA-A2 peptides is 36. For instance, the well-known epitope GILGFVFTL derived from the influenza A matrix protein scores 30. Peptides p1145 (FLFDGSPTYV) and p1258 (FLFDGSPTYVL), both derived from a fatty acid synthase, represent with a length of 10 and 11 amino acids, a typical and a non typical HLA-A2 derived peptide. The sequence of those two peptides is overlapping and only differs in the length (addition of L in p1258). The capability of binding to HLA-A2 differs as expected, as the decapeptide p1145 has a higher affinity to HLA-A2 than peptide p1258. Even though the prediction of binding affinities for MHC-restricted epitopes can provide valuable information about novel peptides, it cannot replace functional assays. Firstly, it is limitated to decapeptides (the score for p1258 with 11 aa length is not predictable) and is not always consistent with experimental binding

affinity studies, as we found a relatively low affinity for peptide p981 compared to the high theoretical score calculated in the database. In these experiments, the optimum of HLA-A2 expression was found for a peptide concentration of 25μg/ml after pulsing for 24 hours. We focused in this study on p1028 as a highly abundant and high affinity peptide for further evaluation of immunogenicity. Further, this peptide is derived from DNA methyl transferase I (DNMT-1), which is a highly abundant protein found in tumors.

Generation of dendritic cells and the induction of CTL.

DCs were separated from leukapheresis products of healthy HLA-A2 positive volunteer blood donors. Immature DCs were obtained by culturing adherent cells with cytokines as explained in Materials and Methods. DCs were phenotyped after 7 days as well as after full maturation. After maturation, DC expressed high levels of the costimulatory molecules B7.1 (CD80) and B7.2 (CD86) as well as MHC class II, Figure 3. In addition, the expression of CD11c and CD83 was enhanced. Mature DC were pulsed with sHLA-A2 derived peptides and used as stimulator cells. Autologous $CD8^+$ T cells were used as responder cells. Peptide p1028-loaded dendritic cells induced clonal expansion of T cells after the first restimulation, whereas no expansion was observed for the lower affinity peptides. These findings indicate that the exogenously added sHLA-A2 derived peptide p1028 is efficiently presented in the MHC class I pathway by dendritic cells, confirming that the peptide is a natural HLA-A2 ligand, and able to induce a T cell response.

Specific CTL reactivity against p1028-pulsed T2 cells

CTL were phenotyped and subsequently tested for lytic activity in a standard ^{51}chromium release assay. As expected, more than 90% of the CTL were CD8+, Figure 4*a*. To test whether those CD8+ T cells specifically lyse p1028 presenting cells, we incubated T2 cells overnight with peptide p1028 and β_2-microglobulin and used them as target cells. CTL lysed peptide p1028 presenting T2 cells, but not unloaded T2 cells, Figure 4*b*. These data demonstrate that peptide p1028 is immunogenic and the generated CTL respond specifically to p1028 expressing cells.

Peptide p1028 specific CTL are able to lyse tumor cells

To investigate whether p1028 specific CTL are able to kill tumor cells, we used the two HLA-A2 positive breast cancer cell lines MCF-7 and MDA-231 as target cells, pulsed T2 cells as positive control and dendritic cells as negative control representing non-oncogenic cells. Even though many tumors downregulate or abrogate their surface MHC expression as a mechanism to evade recognition by T cells, the two breast cancer cell lines studied here expressed HLA-A2 on their surface as analyzed by flow cytometry, Figure 5*a*. If peptide p1028 is potentially presented by membrane-bound MHC molecules, the expression of HLA-A2 should make them susceptible for lysis by p1028 specific CTL. DNMT-1 was degraded to peptide p1028 by those breast cancer cells, as it was displayed in the sHLA-A2 molecule of the transfected breast cancer cells. Peptide p1028 specific CTL efficiently lysed T2 cells pulsed with p1028, as more than 60% lysis was detectable, Figure 5*b*. Interestingly, the CTL were also able to lyse the two breast cancer cell lines, though at a lower percentage than the pulsed T2 cells. The dendritic

cells remained unaffected, indicating that p1028 is physiologically either not or only weakly expressed on non-oncogenic primary cells.

Characterization of CTL by intracellular IFN-γ production to peptide p1028 and tetramer staining

To further characterize the CTL, we tested for their ability to produce IFN-γ to p1028 presenting T2 cells. IFN-γ production was measured intracellularly after 24h stimulation with peptide-loaded or native T2 cells, Figure 6*a*. Significantly more cells, 68%, expressed IFN-γ selectively to p1028 loaded T2 cells, compared to significantly lower cytokine production in response to unloaded T2 cells, 28%. This level of IFN-γ expression can be considered as background, since about the same percentage of intracellular IFN-γ was detected in unstimulated CTL (data not shown), due to their expected high expression of IFN-γ.

To confirm the peptide-dependent cytotoxicity, we stained the CTL with MHC-peptide tetramers. Tetramers were produced commercially expressing either peptide p1028, or an irrelevant peptide, p898. Peptide p898 is derived from an IFN-γ-inducible protein, which we also recovered from sHLA-A2 molecules (15), thus a peptide with the same characteristics as p1028, regarding MHC-restriction. CTL were co-stained with anti-CD8 and tetramer p1028 or p898, respectively. The p1028 tetramer stained 74 % of the CTL, whereas the control tetramer failed to show any significant binding, Figure 6*b*. These results confirm that the generated CTL are highly specific for peptide p1028. Thus, here we describe a novel peptide that induces CTL in vitro, which lyse tumor cells. This peptide provides a novel candidate tumor vaccine.

DISCUSSION

Peptides derived from otherwise physiological proteins, over-expressed in various tumors, have been shown to be targets for immunological response, for example a peptide derived from protein tyrosinase, which was shown to successfully induce the attack and lysis of melanoma cells from cancer patients (10, 11). Identification of peptide p1028, revealed a previously unknown immunogenic HLA-A2 restricted peptide, derived from DNMT-1, using a novel method of identifying peptides eluted from soluble MHC molecules. It has been eluted from the sMHC of ovarian, prostate and breast cancer cell lines.

This study concentrates on novel peptides eluted from soluble MHC, under the assumption that tumor cells evade immune-surveillance by diverting immunogenic peptides into the soluble MHC pool. This might be related to the degradation of proteins to peptides that may have higher affinity for soluble MHC than for membrane-bound MHC, in the course of somatic cional evolution of cancer cells. The lack of the hydrophobic transmembrane domain in the 39kDa heavy chain might cause differences in regards to the binding affinities of peptides and consequently to the folding of the molecule, compared to the intact, and therefore more hydrophobic heavy chain (18). These differences might have a profound influence on the recognition of TAA by T-lymphocytes, as even small variations in the primary peptide sequence can have major impact on the ability to interact with either the MHC or the TcR (25).

Tumor cell lines MDA-231 and MCF-7 produced the 36 kDa as well as the 39 kDa heavy chain, while they lack the ability to release soluble HLA-A2. This phenomenon seems to

be dependent on the type of tumor cell line, as our group has shown previously, that various lymphoid tumor cell lines are able to release soluble MHC (26).

There is evidence, that sHLA-I antigens play a significant role in the modulation of immune responses, such as the ability to block anti-HLA class I antibodies and CTL activity (27). They are able to induce apoptosis in alloreactive CTL, activated $CD8^+$ T cells (28, 29), and, as recently reported, in NK cells through the engagement of CD8 (30). By presenting TAA-derived peptides in the released soluble HLA, T cells will be rendered anergic by TcR stimulation without costimulation, and therefore tolerant towards the TAA.

Here, we selected the most abundant peptides recovered from the sMHC/peptide complex, which were derived from TAA and evaluated their binding affinity to HLA-A2. The peptides presented by an HLA class I molecule are usually 8 to 10 amino acids long with 2 to 3 primary anchor residues that engage the TCR (31). As shown here, the length of peptides eluted from recombinant sHLA-A2 seems not to be different to peptides displayed on membrane bound MHC class I molecules, although a few "outliners" like peptide p1258, with a length of 11 amino acids, were identified.

The peptide further characterized in this study, p1028 (GLIEKNIEL), displays the characteristic anchor residues for HLA-A2, with L at the second position and at position 9. For peptide p1028 high scores were calculated in the database for MHC ligands (24), which was confirmed by our binding affinity studies. It has been reported, that the binding affinity of 9-residue peptides is generally higher than that of longer peptides containing the same core sequences (32), while another group has shown, that some longer peptides can exhibit a similar or even higher affinity for the HLA-A2 molecule,

than do other 9-residue sequences (33). However, we found lower binding affinity of p1258 to HLA-A2 compared to the decapeptide p1145. The highest theoretical score was found for p981, whereas our results indicate a relatively low binding affinity, which was sustained by the lack of ability to induce expansion of T cells in response to p981.

In contrast, the stimulation of $CD8^+$ T cells with p1028 efficiently promoted the expansion of specific CTL. These CTL were capable of killing the peptide loaded T2 cells and showed also lytic activity against the breast cancer cell lines MDA-231, MCF-7, whereas dendritic cells remained unaffected. The lower cytotoxicity against those tumor cell lines compared to loaded T2 cells, may be due to a lower expression of peptide p1028 on membrane bound MHC molecules on those cell lines, which is still sufficient to be recognized by p1028 specific CTL. Peptide-dependent cytotoxicity was confirmed by staining the CTL with the p1028 tetramer.

Many tumors over-express normal proteins, like DNMT-1, thereby modifying the set of self-peptides associated with MHC class I molecules. This phenomenon allows triggering of tumor-specific CTL against self-antigens, but leads to discussion of possible autoimmunity induction and therefore to the question how to increase the specificity to tumor cells. One approach to increase tumor-specificity is to use CTL clones equipped with an NK cell inhibitory receptor, taking advantage of the usually low expression of MHC class I on tumor cells (34). Another limitation to generate tumor-specific T cells against self-peptides in vitro for use in adoptive immunotherapy is the mechanism of diminishing or eliminating self-specific CTL. Since the tolerance to self-antigens is self-MHC restricted, one could generate allorestricted CTL against the peptide of interest.

Those peptides would be self-peptides, but bound by non-self MHC molecules, thereby avoiding the MHC-restricted tolerance to self-antigens (35).

The peptide identified here is derived from DNMT-1, which is overexpressed in tumors and coordinately expressed in normal tissues (36). In malignancy, methylation patterns change, which can lead to genetic instability and the repression of tumor suppressor genes (37). DNMT-1 has already been identified as a target for cancer therapy. Pharmacological DNA methyl transferases inhibitors exerted antitumor effects in *in vitro* and *in vivo* laboratory models, reviewed by Goffin et al (38).

Put together, the wide overexpression of DNMT-1 in various tumors and the finding of a novel immunogenic peptide that can elicit an immune response against tumor cells suggests that this epitope may indeed be suitable for T-cell mediated immunotherapy. We believe that the strategy of recovering peptides from soluble HLA antigens will provide more attractive candidates for vaccination and adoptive T-cell immunotherapy approaches against tumors.

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LEGENDS

Figure 1

Breast cancer cell lines MDA-231 and MCF-7 produce soluble MHC variants. Cell lysates of MDA-231 and MCF-7 were electrophoresed in an SDS-polyacrylamide gel and MHC class I heavy chains detected by immunoblotting using a rabbit anti-human MHC class I polyclonal serum (*a*) or the anti-human soluble HLA-A2 monoclonal antibody 4B5 (*b*). Recombinant sHLA-A2 molecules (sA2 and sA2-Bsp) were used as controls. The 44 kDa intact heavy chain, the 39 kDa spliced product as well as the 36 kDa truncated form of MHC class I are detected in the cell lysates (*a*). Most dominant is the 36 kDa protein in both cell lystes, whereas there is no detectable soluble HLA in the supernatant of both cell lines (*b*).

Figure 2

Peptide p1028 has strong binding affinity to HLA-A2. TAP deficient T2 hybridoma cells were incubated with 25μg/ml sHLA-A2peptide and 15μg/ml β_2-microglobulin at 37°C for 24 h to reconstitute HLA-A2 expression on their cell surface. Expression of HLA-A2 was measured by flow cytometry using the anti-HLA-A2 mAb PA2.1. Highest reconstitution of HLA-A2 was achieved with peptide p1028. Percentage of HLA-A2 expressing cells is indicated by the numbers in each histogram.

Figure 3

Phenotyping of immature and mature dendritic cells (DC). Adherent PBMC were co-cultured in the presence of GM-CSF and IL-4. At day 7, non-adherent cells were harvested and cultured for three days with additional cytokines (TNF-α/IL-1/IL-6). Mature and immature DCs were stained with mAbs for 30 min at 4°C and analyzed by flow cytometry. Characteristically, mature DCs showed enhanced CD80 and CD83 expression.

Figure 4

Effector CTL are able to recognize p1028 loaded T2 cells. CTL were phenotyped before testing their cytotoxicity. The CTL culture contains more than 90% CD8+ T cells, as analyzed by flow cytometry, (*a*). Peptide-pulsed and unpulsed HLA-A2 positive T2 cells were labeled with ^{51}Cr and used as target cells in a 4 h ^{51}Cr-release assay. CTL specifically lysed p1028 pulsed T2 cells, while no significant lysis was detected for the unpulsed T2 cells.

Figure 5

Breast cancer cell lines MDA-231 and MCF-7 are susceptible to lysis by p1028 specific CTL. MDA-231 and MCF-7 cells express HLA-A2 on their cell surface, compared to expression of HLA-A2 on mature dendritic cells (DC). Cells were stained with PA2.1 mAb and a FITC-conjugated secondary antibody, (*a*).

MCF-7, MDA-231 and p1028-pulsed T2 cells were used as target cells in a 4 h ^{51}Cr-release assay. Although peptide-pulsed T2 cells were highly susceptible to CTL killing, the MCF-7 and MDA-231 breast cancer cells were also lysed. No lysis was detectable for

unpulsed dendritic cells used as target cells, indicating that the tumor cells, but not DC display peptide p1028 on their surface MHC, (*b*).

Figure 6

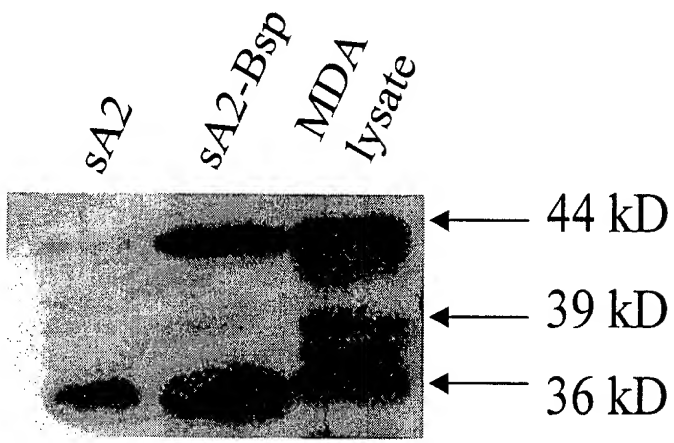
Tetramer binding and intracellular IFN-γ production of p1028 peptide specific CTL.

p1028 specific CTL express intracellular IFN-γ to p1028 peptide-loaded T2 cells. IFN-γ was measured after 24h incubation with either pulsed or unpulsed T2 cells. Cells were stained with anti-CD8, permeabilized and incubated with a PE-conjugated IFN-γ antibody, respectively. Data were analyzed by flow cytometry. 68% of the CTL specifically produced IFN-γ to peptide p1028 loaded T2 cells. Only 28% IFN-γ positive cells were detected in response to unloaded T2 cells (*a*). That level of IFN-γ was, however, also expressed in non-stimulated CTL.

CTL were stained with FITC-conjugated A2.1/p1028 or control A2.1/p898 tetramers and anti-CD8-PE. The p1028 tetramer stains approximately 74% of the CTL, while the irrelevant tetramer showed no significant binding, showing that the generated CTL are highly specific for peptide p1028.

FIGURE 1

a



b

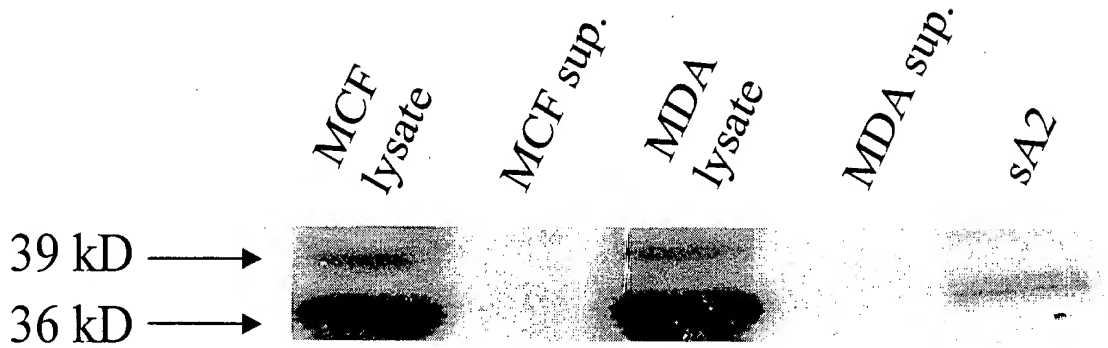


FIGURE 2

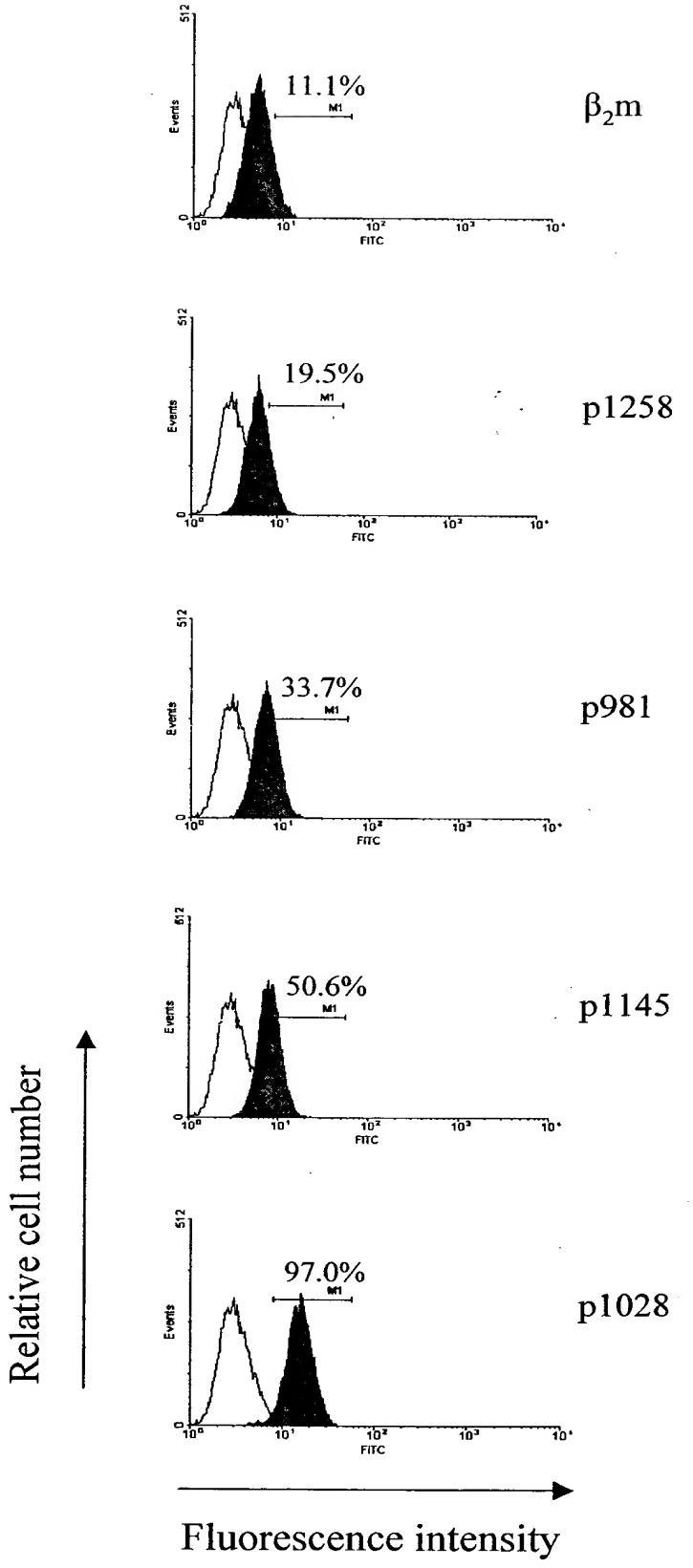


FIGURE 3

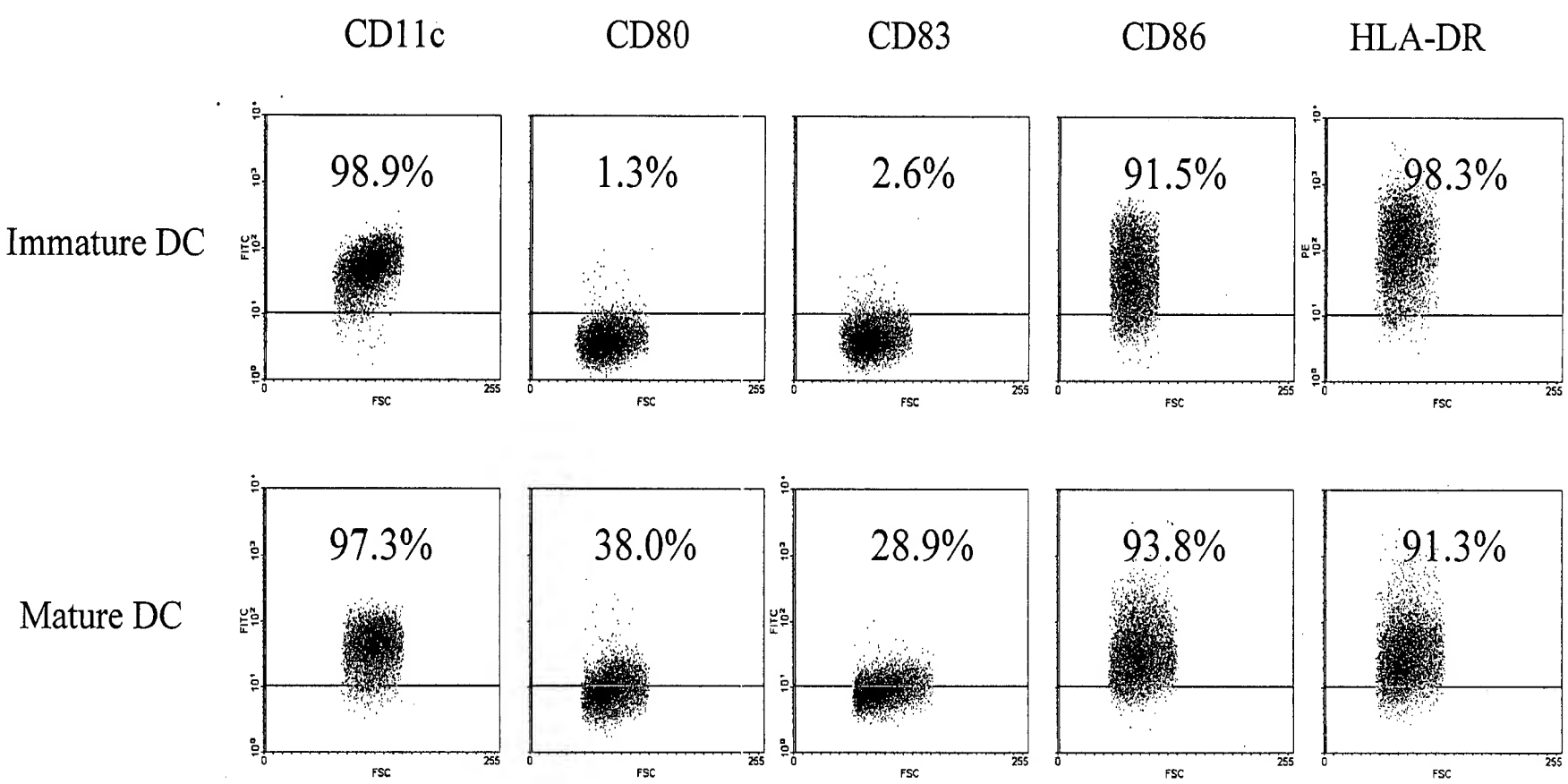
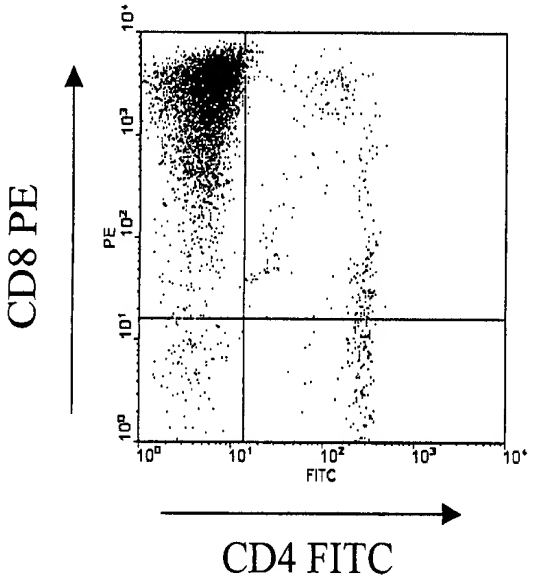

CD11c
CD80
CD83
CD86
HLA-DR
Immature DC
Mature DC
98.9%
1.3%
2.6%
91.5%
98.3%
97.3%
38.0%
28.9%
93.8%
91.3%
FITC
PE
FSC
0
255

FIGURE 4

a



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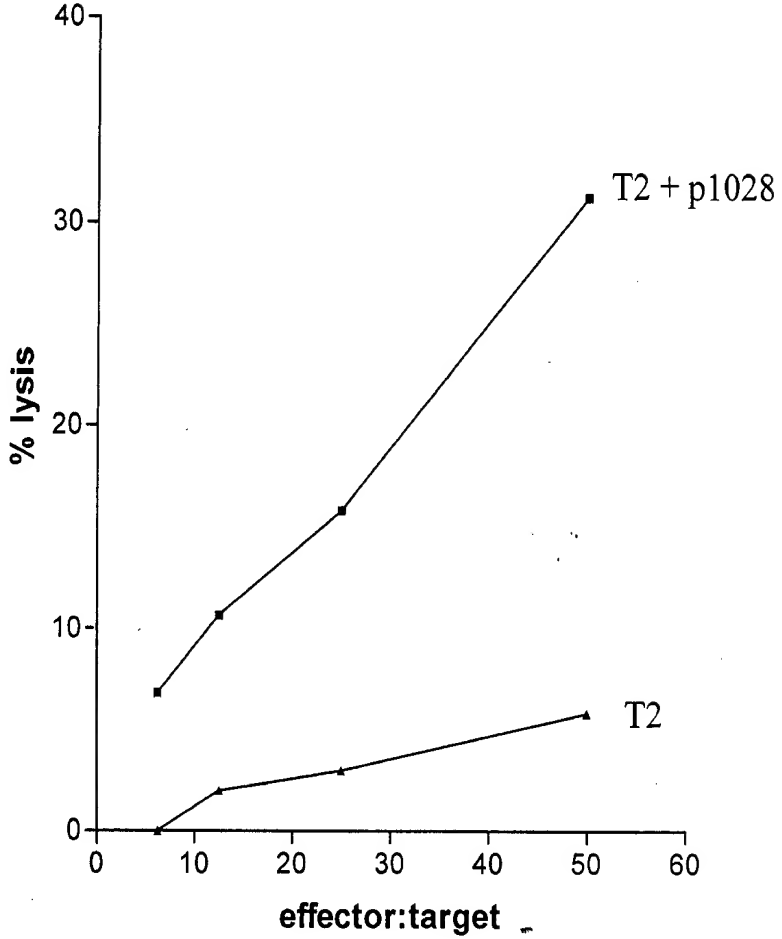
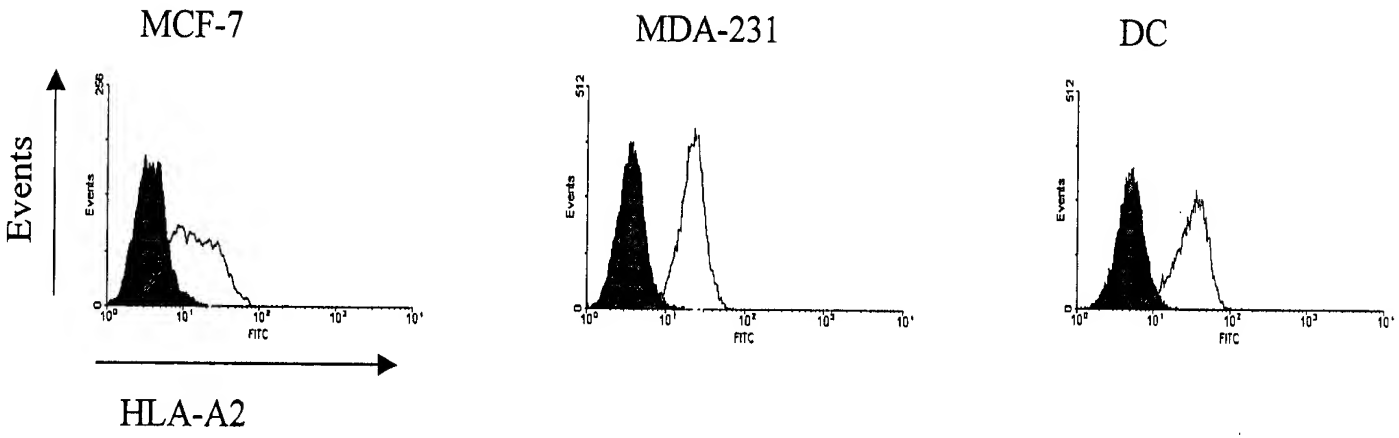


FIGURE 5

a



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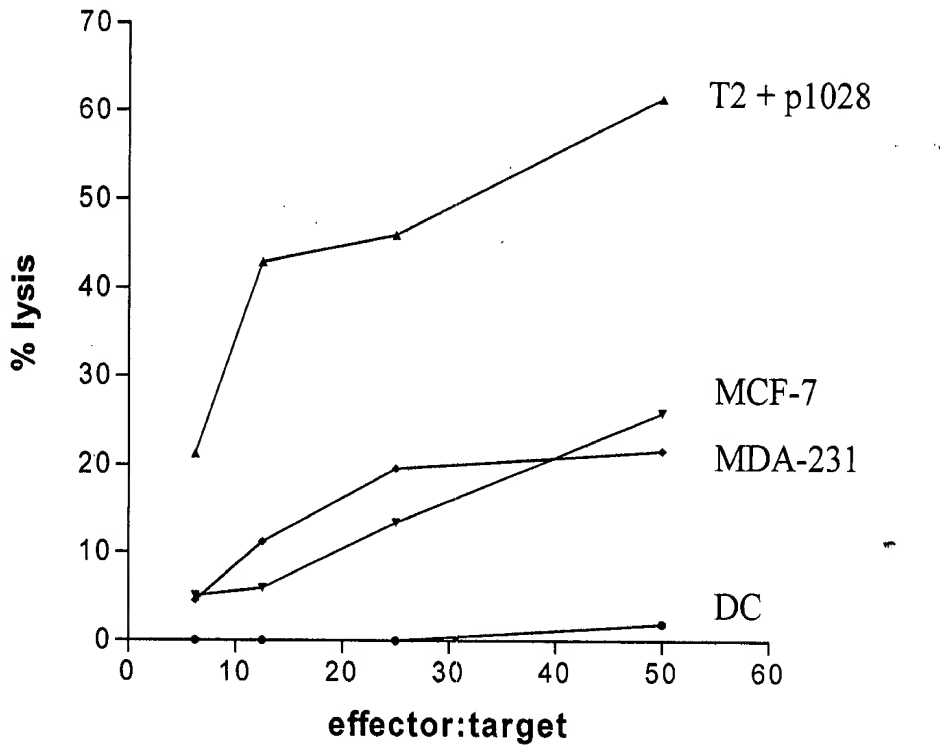
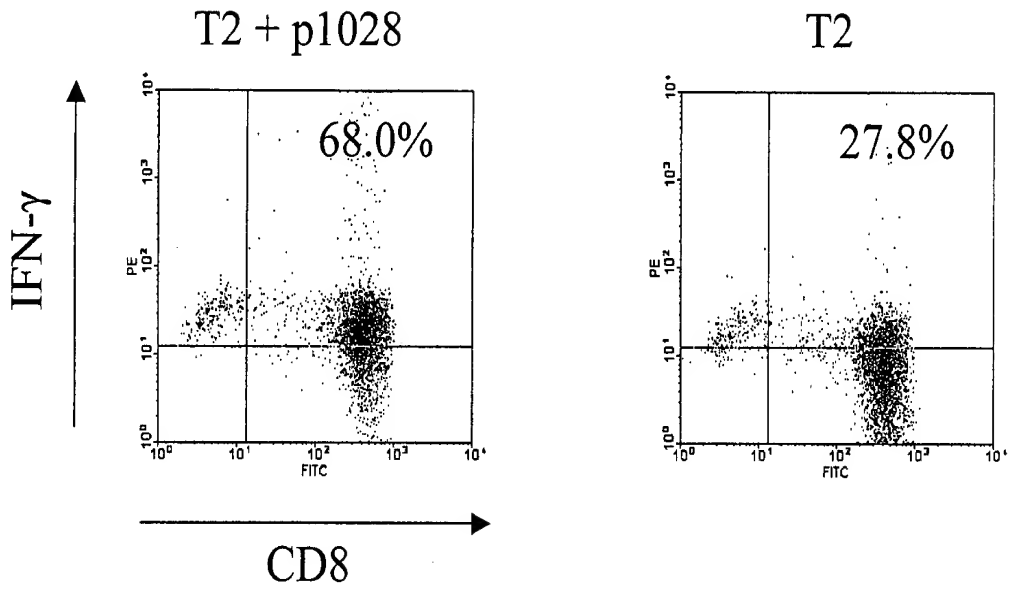


FIGURE 6

a



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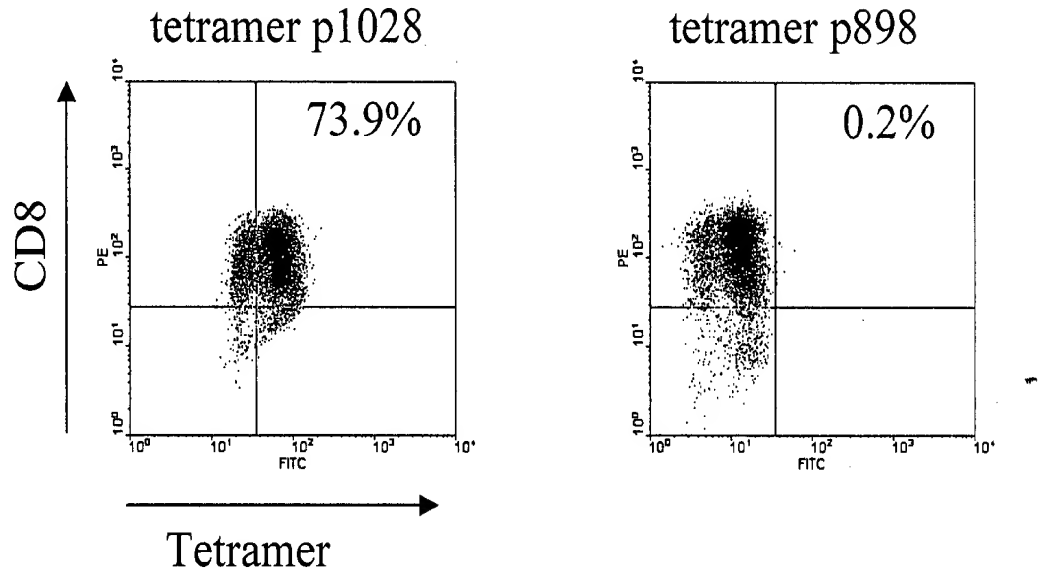

TABLE 1

Peptide	Sequence	Precursor Protein	Score MHC database [25]
p981	SLIGHLQTL	Protein tyrosine phosphatase	32
p1028	GLIEKNIEL	DNA methyl transferase I	28
p1258	FLFDGSPTYVL	Fatty acid synthase	N/A
p1145	FLFDGSPTYV	Fatty acid synthase	23

Arie Admon, Ph.D.

Associate professor of biology

Arie Admon is the head of the Technion Protein Center

Departemnt of Biology

Technion - Israel Institute of Technology

Haifa 32000, Israel

Tel: 04-8293407, 8293446, 8293961

Fax: 04-8225153

E-mail: admon@tx.technion.ac.il

Arie's science talk and Hebrew abstract ('doc' file, open with MS Word)

Courses:

Analytical Biochemistry (of proteins) (138018)

Laboratory course in biochemistry A. (134005).

Analytical Biochemisrty (138018)

This course replaces:

Analytical methods in biochemistry (138075)

Micro-analysis of proteins (138092)

Purification and analysis of Enzymes (138108).

Research Interest

1. Protein Center. A. Admon has established a Center for Protein Engineering and Protein Chemistry at the Department of Biology, Technion. The Center gained national recognition and has been expanded into a National "open" Center for Protein and Peptide analysis which is funded by the Ministry of Sciences and Arts. The main thrust of the Center is of microanalysis of protein and peptides. Main instuments are: HPLCs, 1D, 2D electrophoresis, micro Edman sequencing and mass-spectrometry with MALDI and ESI.

2. Cancer cell surface antigens. Construction and selection of phage-display single-chain human Fv antibodies (scFv) specific for tumor antigens of human cancers. Such human recombinant antibodies specific for human melanoma were selected from a phage-display expression library demonstrating high specificity for a number of melanoma lines and no cross reactivity with other cancer cells or with normal human melanocytes. This project is funded by the Israel Cancer Association, by the Ministry of Health and by Hilman Grant.

3. MHC restricted peptides. Direct chemical analysis of cancer cell MHC restricted peptides presented on human cancer cells. We perform direct analysis by HPLC, mass-spectrometry and Edman sequencing of human cancer MHC class I restricted peptides in order to develop anti-cancer vaccine. This project is funded by the Ministry of Science.

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6. Admon A. Purification of transcription factors by DNA affinity chromatography. Gene Transcription: DNA binding proteins, Essential techniques. Ed: K. Docherty. (1996) pp51-55 John Weley & Sons, Chichester with Bios Scientific Pub. Oxford UK.

Graduate Students:

Ms. Michal Inbar M.Sc. candidate. Identification anitbodies directed at angiogenesis recptors.

Ms. Avital Lev, Ph.D. candidate. Isolation of human cancer antigen by use of the phage-display system.

Mr. Eilon Barnea, Ph.D.. candidate. Direct chemical analysis of MHC peptide antigens from human cancer.

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